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TITLE OF THESIS......STUDIES ON MAMMALIAN

...RIBOSOMES AND PROTEIN

.....SYNTHESIS.........

UNIVERSITY.....OF ALBERTA..........

DEGREE FOR WHICH THESIS WAS PRESENTED.....Ph.D...........

YEAR THIS DEGREE GRANTED......SPRING 1974........

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THE UNIVERSITY OF ALBERTA

STUDIES ON MAMMALIAN RIBOSOMES AND PROTEIN SYNTHESIS

by

© ALBERT JOHN FABER

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA
SPRING, 1974

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *STUDIES ON MAMMALIAN RIBOSOMES AND PROTEIN SYNTHESIS* submitted by ALBERT JOHN FABER in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Date March 26, 1974

To my wife, Patricia.

ABSTRACT

The isolation of active mouse liver run-off 80S ribosomes and their subunits, and conditions necessary for the translation of synthetic and natural messenger RNA in cell-free systems have been studied.

Mouse liver polysomes were incubated in the presence of all components necessary for protein synthesis allowing the run-off of 80S ribosomes. Upon exposure of these 80S particles to 5 mM Mg^{2+}, 500 mM KCl, 70-72% dissociated into ribosome subunits. Treatment of the reaction mixture with 0.1 mM puromycin was sufficient to sensitize the remaining undissociated particles such that complete dissociation was achieved. The 40S and 60S subunits were separated by centrifugation through a convex-exponential sucrose gradient containing 2 mM Mg^{2+}, 300 mM KCl, and recovered from the pooled fractions by centrifugation. The separated subunits were at least 95% pure based on the sedimentation analysis of the particles and RNA extracted. When mixed the subunits spontaneously reassociated to form 80S ribosomes at 5 mM Mg^{2+} in the absence of poly(U), tRNA and supernatant factors. Polyacrylamide gel electrophoresis of ribosomal proteins extracted from 40S and 60S subunits showed electrophoretic patterns distinct from each other. A comparison was made between ribosomal proteins of L5178Y mouse lymphoma and mouse liver; some differences were noted.

The protein synthesizing ability of the purified subunits was tested by measuring poly(U)-dependent incorpora-

tion of [^{14}C]phenylalanine into acid-insoluble material. The 40S and 60S subunits were inactive separately, but when recombined, incorporated 10-15 phenylalanine residues per active 80S ribosome.

A precharged tRNA incorporation system was developed as an assay to measure peptide chain elongation factor and protein initiation factor activity. Mouse liver and *E. coli* [^{14}C]Phe-tRNA functioned as substrates in poly(U)-directed polyphenylalanine synthesis. The reaction was linear for 10 min with 50% of the substrate being incorporated into hot acid-insoluble material. Elongation factors EF_1 and EF_2 were partially purified by $(NH_4)_2SO_4$ fractionation and Sephadex G-100 chromatography. Ribosomes, EF_1, EF_2, GTP and poly(U) were found to be essential for incorporation at 7.5 mM Mg^{2+}.

The requirements for phenylalanine incorporation at 3.5 mM Mg^{2+} in the presence and absence of ribosome wash was studied. Addition of crude 0.5 M KCl ribosome wash reduced the Mg^{2+} optimum for polyphenylalanine synthesis from 7.5 mM to 3.5 mM Mg^{2+}, while stimulating incorporation at 3.5 mM Mg^{2+} as much as 9-fold. The response of four different preparations of mouse liver ribosomes to ribosome wash and also the substrate specificity of *E. coli*, yeast, and mouse liver tRNA was tested by this assay. Crude 0.5 M KCl ribosome wash was fractionated by DEAE-cellulose chromatography, and the fractions assayed for the ability to support phenylalanine incorporation at 3.5 mM Mg^{2+}.

Highest stimulation was achieved when fractions I, VI and VIII, which eluted at 0.05 M KCl, 0.18-0.25 M KCl, and 0.31-0.38 M KCl, respectively, were present.

Polysomal RNA was extracted from membrane-bound and total polysomes by two different methods, and translated in a homologous mouse liver cell-free system. Further purification of RNA by cellulose chromatography and sucrose density-gradient centrifugation was examined. A cell-free incorporation system utilizing run-off 80S ribosomes was developed which was strictly dependent upon added protein initiation factors and mRNA for activity. The optimum concentration of Mg^{2+}, KCl, ribosomes, ribosome wash, and polysomal RNA for protein synthesis was determined. Sucrose density-gradient analysis of the reaction products revealed formation of new polysomes containing up to ten 80S ribosomes. Translation of exogenous mRNA was found to be inhibited 80% by 1×10^{-5} M aurintricarboxylic acid; a concentration which had no effect on endogenous incorporation. Analysis of the cell-free products by polyacrylamide gel electrophoresis and immunoprecipitation showed that newly formed albumin could account for up to 8% of the total protein synthesized.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my supervisor, Dr. T. Tamaoki, for his guidance and encouragement during the course of these studies. He introduced me to the field of molecular biology and provided many opportunities for me to attend scientific meetings; for this I am grateful.

I am also thankful for the assistance and interest shown in my work by Dr. A.R.P. Paterson, Dr. G.A. LePage, Dr. J.S. Colter and other members of the McEachern Cancer Research Unit and Department of Biochemistry.

During these studies I have had helpful discussions with other members of our research group, notably Dr. C.N. Murty and Dr. S. Miall. I also thank Mr. K. Thomas and Miss I. Schindler for their assistance in providing enzyme and ribosome wash fractions on numerous occassions.

I have been the holder of a National Cancer Institute of Canada Fellowship and have also received financial support from the University of Alberta Cancer Research Unit, for which I am grateful.

Lastly, I wish to thank my wife for the many hours spent typing this thesis, to her parents for their encouragement, to Mrs. Davis for her generosity and to my mother and late father for embarking me on a scientific career.

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LIST OF ABBREVIATIONS

Phe	phenylalanine
Leu	leucine
PEP	phosphoenolpyruvate
PK	pyruvate kinase
ATP	adenosine-5'-triphosphate
GTP	guanosine-5'-triphosphate
mRNA	messenger ribonucleic acid
tRNA	transfer ribonucleic acid
poly(U)	polyuridylic acid
poly(A)	polyadenylic acid
oligo (dT)	oligothymidylic acid
DEAE-cellulose	diethylaminoethyl-cellulose
EF_1	peptide chain elongation factor 1
EF_2	peptide chain elongation factor 2
ATA	aurintricarboxylic acid
TCA	trichloroacetic acid
SDS	sodium dodecyl sulfate
DOC	deoxycholate
β-ME	β-mercaptoethanol
DTE	dithioerythritol
EDTA	ethylenediaminetetraacetic acid
m, μ, p	milli, micro, pico (10^{-3}, 10^{-6}, 10^{-12})

CHAPTER I

GENERAL INTRODUCTION

The mechanism of protein synthesis has been a topic of extensive investigation during the past two decades. Studies on protein biosynthesis *in vitro* began when Siekevitz (1952) prepared rat liver microsomes which were capable of incorporating labeled alanine into protein. Hoagland and coworkers (1956, 1957) using a rat liver cell-free system found that ATP, a supernatant protein (i.e. amino-acyl-tRNA synthetase), and a low molecular weight RNA (i.e. tRNA) were essential for incorporation of amino acids into protein.

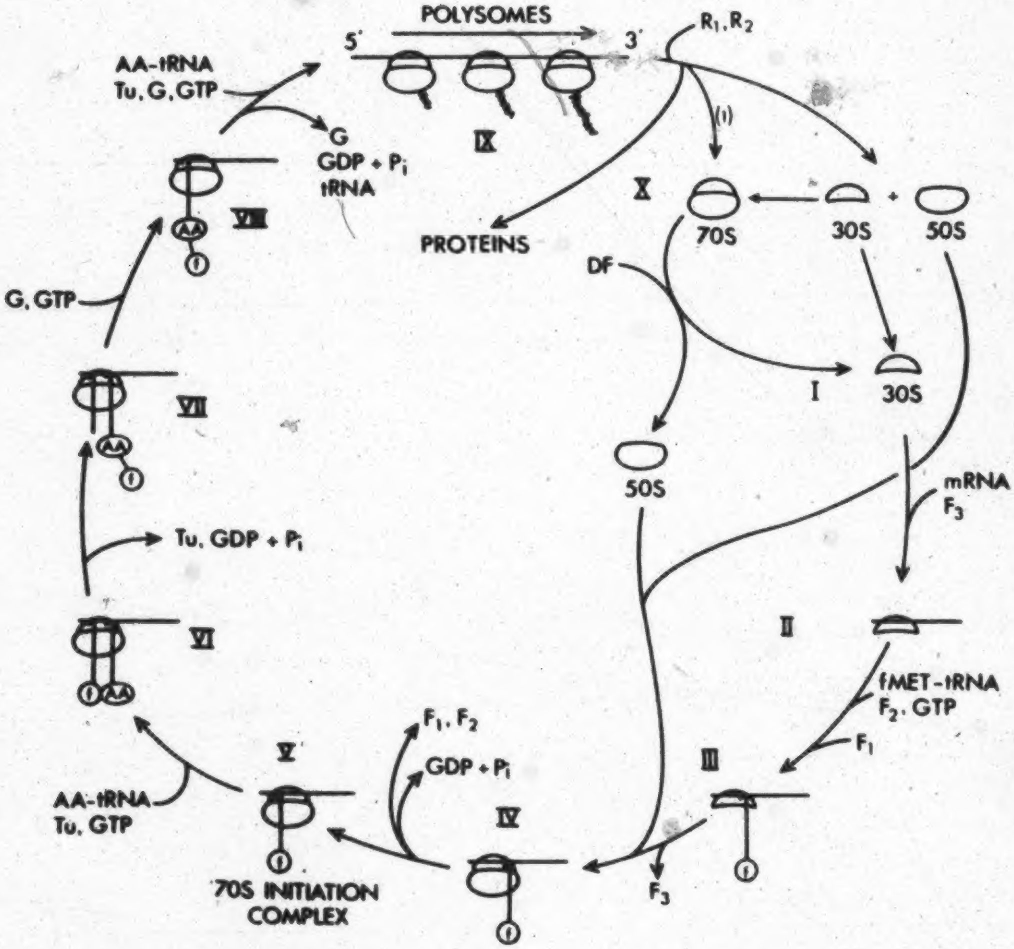
Ribonucleoprotein particles composed of approximately equal amounts of RNA and protein, later called ribosomes, were identified as the site of protein synthesis (Palade, Siekevitz, 1956; Siekevitz, Palade, 1958). In 1958 Tissières and Watson isolated ribosomes from *Escherichia coli* (*E. coli*) and found that 90% of the cellular RNA was present as ribosomal RNA. At this time it was not clear how information contained in DNA cistrons was transferred to the cytoplasm where ribosomes could assemble amino acids into proteins. Ribosomal RNA was a prime candidate for the template RNA (Crick, 1958) although the homogeneity of size and composition did not seem to reflect the range of size of polypeptides synthesized.

In 1961 Jacob and Monod proposed that each DNA cistron acts as a template for the synthesis of a messenger RNA molecule which contains the amino acid sequence information encoded in its nucleotides. The theory was soon supported with evidence (Brenner, Jacob, Meselson, 1961) yet the complete realization of the messenger RNA concept did not occur until the genetic code was elucidated (Gamow, 1954; Crick *et al.*, 1961; Nirenberg, Leder, 1964; Morgan, Wells, Khorana, 1966).

Knowledge of the reactions which occur during peptide bond formation have primarily come from bacterial cell-free amino acid incorporation systems, the majority of which are *E. coli*; however, mammalian cell-free systems have also made a contribution. Several review articles have been written on the interaction of the ribosome and supernatant factors during initiation, elongation and termination of protein synthesis (Cold Spring Harbour Symp. Quant. Biol., 1969; Lengyel, Soll, 1969; Lucas-Lenard, Lipmann, 1971; Haselkorn, Rothman-Denes, 1973).

The overall process of protein synthesis can be schematically represented by the ribosome cycle depicted in Figure 1. This cycle was drawn based on the two site tRNA binding hypothesis but does not exclude the possibility that there are more than two binding sites.

Figure 1. Schematic representation of protein synthesis and the ribosome cycle of *E. coli*. The ribosome cycle depicts the movement of ribosomes through the three stages of protein synthesis, initiation (I-V), elongation (V-IX), and termination (IX-X). The 30S subunits obtained either from resting 70S particles by the action of dissociation factor (DF) or from free 30S subunits, interacts with mRNA and F_3 (IF-3), an initiation factor specific for natural mRNA (Step II). DF may be one of the initiation factors, possibly F_3. N-formylmethionyl-$tRNA_F$ (fMet-tRNA) in the presence of initiation factor F_2 (IF-2) and GTP then binds to the initiator codon AUG (Step III). For convenience fMet-tRNA is shown bound in the "A" or aminoacyl-tRNA acceptor site and subsequently translocates to the "P" or peptidyl-tRNA site although the precise binding site is uncertain. The initiation factor F_1 (IF-1) catalyzes the release of F_2 and possibly assists in the binding of the 50S subunit to the complex. Step V is known as the "70S initiation complex". Elongation begins with the binding of aminoacyl-tRNA (aa-tRNA) to the vacant A site specified by the second codon (Step VI). This binding requires GTP and Tu, the aa-tRNA binding factor. Peptidyl transferase, located on the 50S subunit catalyzes peptide bond formation between formylmethionine and the second amino acid (Step VII). Translocation then occurs involving the removal of the deacylated tRNA, the coordinate movement of the ribosome by one codon relative to mRNA, and the transfer of fMet-aa-tRNA from the A to the P site (Step VIII). The translocation factor G and the hydrolysis of GTP mediate this process. Step VI and VIII are repeated until a termination codon (UAA, UAG, UGA) is reached. Release factors, R_1 and R_2, promote the cleavage of the ester bond between the polypeptide and tRNA bound in the P site, thereby releasing the completed protein. If no more codons are to be translated then the ribosome disengages from the mRNA either as free 70S ribosomes (1) or as subunits (2). The exact route of this disengagement remains unclear at this time.


POLYSOMES
5′
3′
R₁, R₂
AA-tRNA
Tu, G, GTP
G
GDP + Pᵢ
tRNA
IX
(i)
X
70S
30S
+
50S
PROTEINS
VIII
AA
f
G, GTP
DF
I
30S
VII
AA
f
50S
mRNA
F₃
Tu, GDP + Pᵢ
VI
f
AA
II
fMET-tRNA
F₂, GTP
F₁
F₁, F₂
V
GDP + Pᵢ
III
AA-tRNA
Tu, GTP
IV
f
f
f
F₃
70S INITIATION
COMPLEX

Several areas of the cycle are not as well understood as others. The relationship between the three initiation factors which are found to work cooperatively are not fully known. Recent results suggest that the dissociation factor (DF) required to maintain free 30S subunits, may in fact be an activity associated with initiation factor F_3 (IF-3) (Sabol *et al.*, 1970; Subramanian, Davis, 1970; Kaempfer, 1970; Kaempfer, 1971). Other areas of uncertainty include the initial binding site of initiator tRNA on the small subunit and also the mechanism by which completed polypeptides are released from the ribosome.

Lack of study in the eucaryote system relates primarily to the difficulty of isolating highly active and well characterized ribosomes and ribosome subunits for cell-free studies. Figure 2 shows a tentative ribosome cycle drawn to illustrate mammalian protein synthesis. The overall pattern appears similar to that of procaryotes; however, many of the steps are not well established and for this reason were drawn in summary rather than as intermediates.

Similarities between procaryote and eucaryote protein synthesis include the initiation complex being formed on the small subunit and the requirement of several initiation factors with one specific for natural mRNA, GTP for aminoacyl-tRNA binding and peptidyl-tRNA translocation, and protein factors for elongation and termination. Differences that have been noted in eucaryotes include a larger ribosome particle and an initiator tRNA that does not require methionine to be formylated.

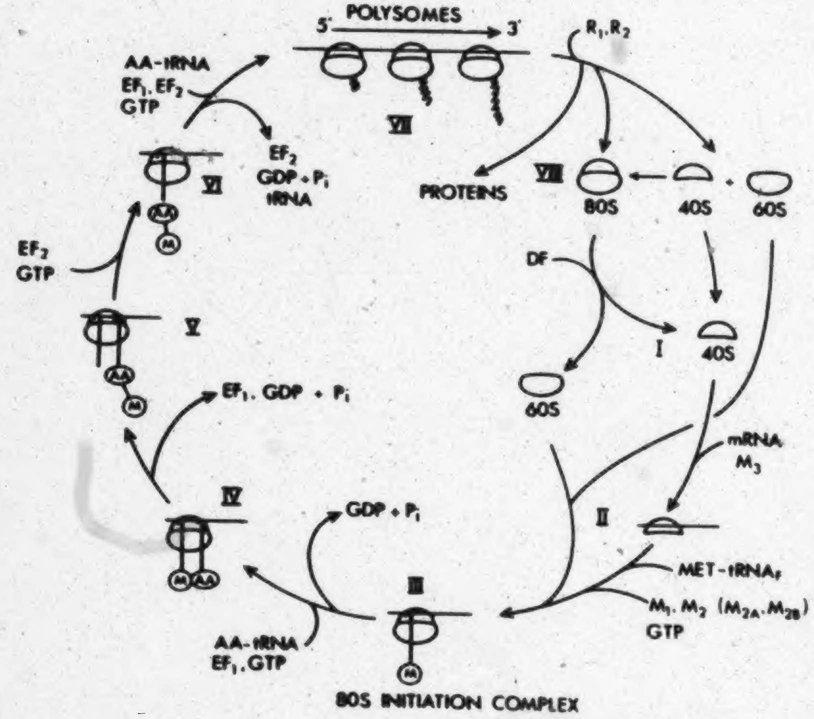

POLYSOMES
5'
3'
R_1, R_2
AA-tRNA
EF_1, EF_2
GTP
VII
EF_2
GDP + P_i
tRNA
VI
PROTEINS
VIII
80S
40S
60S
EF_2
GTP
DF
V
I
40S
60S
EF_1. GDP + P_i
mRNA
M_3
IV
GDP + P_i
II
MET-$tRNA_f$
III
M_1, M_2 (M_{2A}, M_{2B})
GTP
AA-tRNA
EF_1, GTP
80S INITIATION COMPLEX

Figure 2. Tentative schematic representation of protein synthesis and the ribosome cycle in eucaryotes. Similar to the procaryote ribosome cycle (Fig. 1) there are three stages of protein synthesis, initiation (I-III), elongation (III-VI) and termination (VII-VIII). 40S subunit interacts with mRNA and M_3, an initiation factor specific for natural mRNA (Step II). 80S initiation complex is formed by the addition of Met-$tRNA_F$, M_1, M_2, GTP and the 60S subunit (Step III). The sequence of addition and release of factors is not certain. Chain elongation and termination are believed to occur by a mechanism similar to that of procaryotes except EF_1 replaces Tu:Ts and EF_2 replaces G.

The purpose of this research was to develop a method for the isolation of active mammalian ribosomes and their subunits, to determine the optimum conditions required for translation of both synthetic and natural mRNA, and to identify the cell-free product directed by exogenous liver mRNA.

Isolation of mouse liver 40S and 60S ribosome subunits and the criteria used to determine the quality and purity of the preparation are described in Chapter II. The biosynthetic activity of the subunits and run-off 80S ribosomes were examined using the "Nirenberg" and "precharged tRNA" incorporation systems (Chapter III). The precharged tRNA incorporation system with partially purified elongation factors EF_1 and EF_2 was designed and used to assay initiation factor activity in the crude and DEAE-cellulose fractionated ribosome wash. Having established conditions required for the detection of initiation factor activity when using poly(U) as template, it remained to examine the *in vitro* translation of natural mRNA. Mouse liver mRNA extracted from membrane-bound and total polysomes was translated in a homologous cell-free amino acid incorporation system utilizing run-off 80S ribosomes.

The chapters have a format similar to manuscripts in that each contains an introduction, materials and methods, results and discussion. References and methods relevant to the topic of each chapter are described therein.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF MOUSE LIVER RUN-OFF 80S RIBOSOMES AND THEIR SUBUNITS

A. Introduction

Involvement of ribosomal subunits in the initiation of protein synthesis has been well documented by studies using *Escherichia coli* cell-free systems (Pestka, Nirenberg, 1966; Mangiorotti, Schlessinger, 1967; Eisenstadt, Brawerman, 1967; Hille *et al.*, 1967; Nomura, Lowry, 1967; Kaempfer, 1968). An advantage of the bacterial system is the ease with which ribosomal subunits can be obtained. In order to study the mechanism of protein synthesis in a mammalian system, sufficient ribosome subunits must be available. Mammalian 80S ribosomes are stabilized to a much greater extent than bacterial 70S ribosomes by bound peptidyl-tRNA and mRNA (Martin *et al.*, 1969; Lawford, 1969; Blobel, Sabatini, 1971). This inherent stability of 80S ribosomes makes it difficult to prepare active mammalian subunits by ordinary dissociation techniques. Methods that have been used to date include the use of high concentrations of KCl, (Martin, Wool, 1968; Martin *et al.*, 1969; Martin, Wool, 1969; Rao, Moldave, 1969; Terao, Ogata, 1970), *in vitro* incubation of polysomes (Falvey, Staehelin, 1970) and the use of puromycin to release the nascent peptide chains (Lawford, 1969; Blobel, Sabatini, 1971).

This chapter describes a method for the preparation of ribosomal subunits from mouse liver in which polysomes are initially incubated in an *in vitro* amino acid incorporation system allowing run-off 80S ribosomes. Those ribosomes that still contain peptidyl-tRNA are sensitized to KCl by a brief exposure to a low concentration of puromycin. The advantage of this method is that with the preincubation of polysomes the amount of puromycin necessary to remove residual peptidyl-tRNA is much smaller than required otherwise (Lawford, 1969; Blobel, Sabatini, 1971). The mouse liver ribosome subunits produced by this method were found to be intact and capable of spontaneously reassociating to form active 80S particles.

B. Materials and Methods

All routine chemicals used were of certified quality. Sterile conditions were used in the preparation of buffers and handling of glassware used in polysome preparation. Ultra pure RNase-free sucrose purchased from Schwarz/Mann was used to prepare buffers and gradient solutions. ATP, GTP, creatine phosphate, creatine phosphate kinase were products of Sigma Chemical Co. Puromycin dihydrochloride and twenty amino acids were purchased from Nutritional Biochemicals Co. [^{14}C]phenylalanine, 355 mCi/mmole; ^{14}C-protein hydrolysate, 57 mCi/milli atom carbon were purchased from Amersham/Searle.

Buffers: All buffers used in the various preparations contain 20 mM Tris-HCl (pH 7.4), 6 mM β-mercaptoethanol (β-ME), 0.25 mM dithioerythritol (DTE) and 10% glycerol in addition to $MgCl_2$ and KCl at concentrations indicated below.

Buffer A: 5 mM Mg^{2+} - 100 mM KCl
Buffer B. 2 mM Mg^{2+} - 100 mM KCl
Buffer C: 2 mM Mg^{2+} - 300 mM KCl

Preparation of polysomes

Polysomes were prepared by a modification of the method of Falvey and Staehelin (1970). Female Swiss albino mice were killed by decapitation, the livers removed, rinsed in buffer A containing 0.3 M sucrose and homogenized with the same buffer (2.5 ml/g of liver) in a Potter-Elvehjem homogenizer with 5 strokes of a loose-fitting Teflon pestle. During homogenization magnesium bentonite (4 mg/9 g liver) prepared as described by Petermann and Pavlovec (1963) was added. The homogenate was centrifuged at 12,000 x g (av) for 10 min and the top two-thirds of the supernatant adjusted to 1% Triton X-100 or 1% DOC. This was layered over a discontinuous gradient (13 ml per gradient) containing 10 ml of each of 0.7 M and 2.0 M sucrose in buffer A. The gradients were centrifuged in a SW 27 rotor at 82,500 x g (av) for 24 hours. The supernatant was carefully removed and the pellets gently resuspended in buffer A and stored in small amounts in liquid nitrogen.

Determination of protein and RNA

Protein was determined by the method of Lowry *et al.*, (1951) or by absorbancy at 260 and 280 nm. Bovine serum albumin was used to construct a standard concentration curve. RNA was determined by measuring the absorbance assuming 20 A_{260} units was equivalent to 1 mg RNA.

Preparation of enzyme fraction

(1) S-200 protein.

Livers were homogenized in a modified buffer A containing 5 mM Mg^{2+} - 25 mM KCl. After high speed centrifugation in a Spinco 60 Ti rotor (233,000 x g (av), 120 min), the top two-thirds of the post-ribosomal supernatant (S-200) was collected and dialyzed overnight at 4°C against buffer A. The S-200 protein was stored in glass vials (0.1-0.2 ml each) in liquid nitrogen.

(2) pH 5 enzymes.

The S-200 fraction was diluted three-fold with 1 mM DTE and slowly adjusted to pH 5.2 with 1N acetic acid. The precipitate was collected by centrifugation at 12,000 x g (av) for 10 min, dissolved in buffer A containing 50 mM Tris-HCl (pH 7.8) and stored in liquid nitrogen.

Preparation of run-off 80S ribosomes and ribosome subunits

Run-off 80S ribosomes were prepared by a modification of the methods of Falvey and Staehelin (1970) and Lawford (1969). Polysomes were incubated in a cell-free amino acid incorporation system containing the following per ml: 30 A_{260} units polysomes, 0.05 μmoles each of the 20 amino acids, 4 mg pH 5 enzyme protein, 2 mg S-200 protein, 1 μmole ATP, 0.3 μmole GTP, 10 μg creatine phosphate, 50 μg creatine phosphokinase, 50 μg mouse liver tRNA, 20 μmole Tris-HCl (pH 7.4), 100 μmole KCl, 6 μmole β-ME, 0.25 μmole DTE and 4 μmole $MgCl_2$. The reaction mixture was incubated at 37^0 for 30 min and 10 mM puromycin (pH 6.5) was then added to a final concentration of 0.1 mM. This mixture was cooled in ice and 2 M KCl was added with stirring to give a final concentration of 3 mM Mg^{2+}-500 mM KCl. Further incubation at 37^0 for 10 min resulted in a preparation containing only ribosomal subunits. Upon dilution of the reaction mixture to 5 mM Mg^{2+}-100 mM KCl the subunits were reassociated to form 80S ribosomes (run-off 80S). They were sedimented by centrifugating a 20 ml sample over 7 ml of 1.5 M sucrose in buffer A in a Spinco Type 60 Ti rotor at 176,000 x g (av) for 4 hours. The 80S ribosome pellets were resuspended in buffer A and stored in small amounts in liquid nitrogen.

Ribosomal subunits were separated by layering 5 ml of the puromycin-treated 3 mM Mg^{2+}-500 mM KCl reaction mixture onto a 30 ml 0.3-1.0 M convex-exponential sucrose gradient in buffer C. The sample was centrifuged in a Spinco SW 27 rotor at 82,500 x g (av) for 14 hours and the gradient was displaced with 60% sucrose which was pumped into the bottom of the tube. The effluent was conducted into a flow cell attached to a Gilford Model 2000 spectrophotometer for continuous monitoring and recording of absorbance at 260 nm. The appropriate fractions were pooled and the subunit recovered by centrifugation in a Spinco Type 65 rotor at 160,000 x g (av) for 9 hours. Further purification of the 60S subunits was accomplished by recentrifugation through a 0.3-1.0 M convex-exponential sucrose gradient in buffer A. The center portion of the 60S peak was pooled and the subunits recovered as described above. The subunit pellets were then resuspended in buffer B, and stored at a concentration of 50-60 A_{260} units per ml in small amounts (0.05-0.1 ml) in liquid nitrogen without appreciable loss of activity for 6 months.

Sucrose density-gradient analysis

Ribosome samples of 0.05-0.10 ml containing 0.5-1.0 A_{260} units were layered on the top of 4.5 ml 10-20% linear or 10-34% (0.3-1.0 M) convex-exponential sucrose gradients made up with the same buffer as the samples except that β-ME, DTE and glycerol were not included. The samples were centrifuged in a Spinco SW 50.1 rotor at the speed indicated in each figure. The gradients were analyzed for absorbance at 260 nm from the top as described above.

Determination of percent dissociation of 80S ribosomes

After analyzing a sample of ribosomes by sucrose density-gradient centrifugation as described above, the area under the absorbance peaks was measured by a planimeter. The percent dissociation of 80S ribosomes into subunits was then calculated from the ratio of subunit area to total area.

Analysis of ribosomal RNA by sucrose density-gradient centrifugation

Sodium dodecylsulfate was added to the ribosome samples to a final concentration of 0.1% to dissociate the protein from ribosomal RNA (Gilbert, 1963). After incubation at 37° for 3 min the samples were cooled in ice, centrifuged at 3000 x g (av) for 5 min, and a portion of the supernatant analyzed on a 10-20% sucrose gradient made up with 20 mM Tris-HCl (pH 7.4), 1 mM Mg^{2+}, and 50 mM NaCl. Centrifugation was at 48,000 rpm for 2.5 hr in a Spinco SW 50.1 rotor at 4°. The gradients were analyzed for absorbance at 260 nm as described previously.

Analysis of ribosomal proteins by polyacrylamide gel electrophoresis

An equal volume of 4 M LiCl - 8 M urea was added to a sample of 5 A_{260} units of ribosomes and incubated overnight at 4° (Spitnik-Elson, 1965). The RNA was removed by centrifugation at 12,000 x g (av) for 10 min. The supernatant containing the ribosomal proteins was dialyzed overnight against 6 M urea containing 20 mM Tris-HCl (pH 7.4) and 6 mM β-ME. Electrophoresis was carried out in 10% polyacrylamide gels (0.5 x 9 cm) containing 6 M urea at pH 4.5 at 4 mA per gel. The proteins were stained with 1% Amido Black in 7.5% acetic acid for 1 hr and then destained electrophoretically.

C. Results

1. Conditions required for preparation of run-off 80S ribosomes and subunits

(a) Polysome "run-off"

Polysomes containing 8-15 ribosomes are needed to make the run-off of 80S ribosomes as efficiently as possible. The degradation of polysomes during homogenization was reduced by using slow grinding speeds and by the use of magnesium bentonite as an RNase inhibitor. Coarse magnesium bentonite, prepared by the method of Petermann (1963),was found to increase the yield of large polysomes and also resulted in a 50% decrease of 80S particles in the preparation as assayed by sucrose density-gradient analysis. Nine distinct bands of polysomes could be resolved by zone velocity sedimentation using a 0.3-1.0 M convex-exponential sucrose gradient (Figure 3A).

The kinetics of polysome "run-off" was examined by incubating polysomes with all the components required for protein synthesis and analyzing samples at various times by

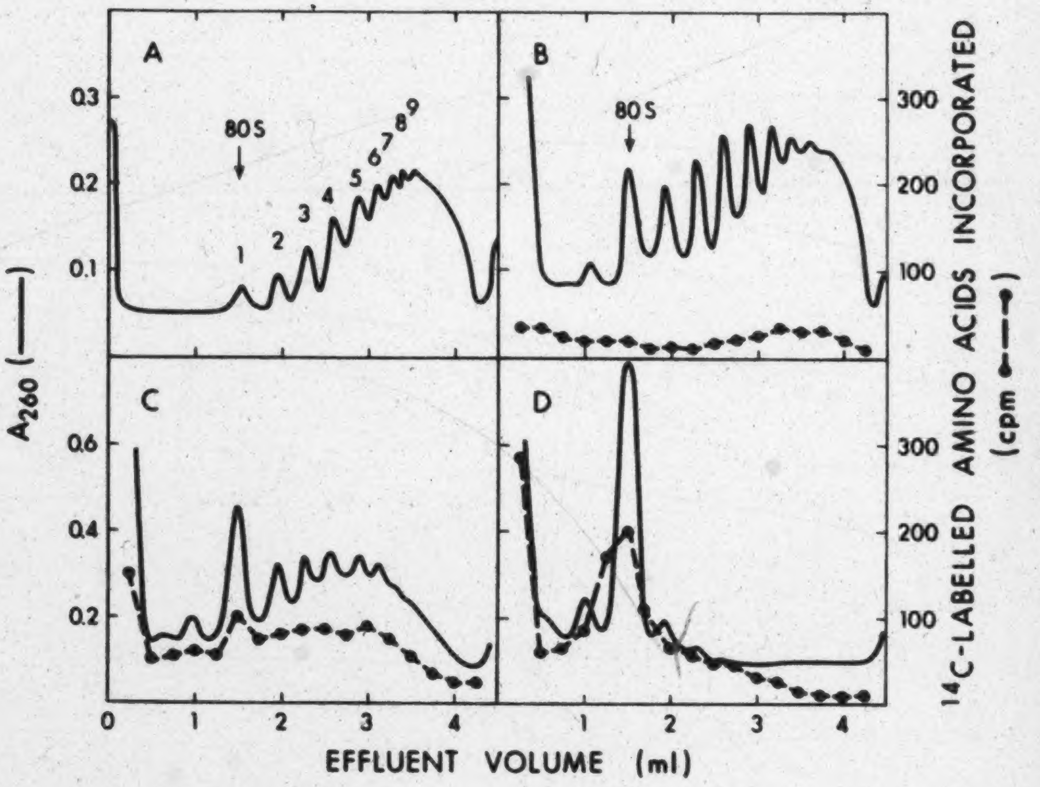


Figure 3. Sucrose density-gradient analysis of mouse liver polysomes incubated in a cell-free amino acid incorporation system. Polysomes were incubated with ^{14}C-protein hydrolysate under the conditions used for the preparation of run-off 80S ribosomes as described in Materials and Methods. Approximately 1.0 A_{260} unit of polysomes was analyzed per gradient. Convex-exponential sucrose gradients (0.3-1.0 M) were made up with 20 mM Tris-HCl (pH 7.4), 5 mM Mg^{2+} and 100 mM KCl. Centrifugation was in a Spinco SW 50.1 rotor at 40,000 rpm for 30 min at 4°. Fractions (0.25 ml) were collected and hot TCA-insoluble radioactivity determined as described in Materials and Methods.

A. Control, polysomes only.
B. Polysomes incubated at 0° for 30 min in the presence of all components required for protein synthesis.
C. Same as B except incubated at 37° for 5 min.
D. Same as B except incubated at 37° for 15 min.

sucrose density-gradient centrifugation (Figure 3). ^{14}C-protein hydrolysate was included in the reaction mixture thereby allowing measurement of hot TCA-insoluble radioactivity in the gradient fractions. During incubation the polysomes rapidly shift from a large to a smaller size with a corresonding increase in the amount of 80S particles (Fig. 3B and 3C). The highest radioactivity was initially in the ~~polysome~~ region and was observed to shift towards the smaller polysomes with time. After 15 min incubation at 37°, polysome "run-off" was virtually complete with only disomes remaining. Continued incubation for 30, 45 and 60 min gave the same pattern as the 15 min sample. Exposure of a 60 min sample to 3 mM Mg^{2+}-500 mM KCl resulted in the dissociation of the 80S ribosomes into subunits. Sucrose gradient analysis indicated that the labeled peptides were bound exclusively to the 60S subunits. Presumably these peptides were short enough that they did not hinder dissociation of the ribosome particle into subunits.

(b) Dissociation of run-off 80S ribosomes into subunits

A criterion for the effectiveness of run-off is the extent of dissociation of 80S ribosomes into subunits that occurs on increasing the salt concentration to 300-500 mM KCl (Lawford, 1969).

Table 1 shows the relationship between the conditions of polysome incubation, the ionic strength at the dissociation step and the extent of dissociation achieved. Polysomes incubated at 37° in the presence and absence of components that support protein synthesis were found to contain 12% and 5% subunits, respectively, when analyzed by sucrose gradient centrifugation under normal ionic conditions (Expt. 1). Incubation of polysomes alone at 37° followed by exposure to high KCl resulted in one-third of the ribosomes dissociating into subunits (Expt. II). However, if this incubation was carried out in the presence of an energy-generating system and supernatant factors, then 70-72% of the run-off 80S ribosomes dissociated into subunits (Expt. III).

The remaining 80S ribosomes and disomes are resistant to dissociation and most likely contain peptidyl-tRNA and mRNA. These particles do not represent run-off 80S ribosomes but rather "stuck 80S ribosomes". This was shown to be the case by recovering these ribosomes from a gradient, treating them with puromycin and finding them to be sensitive to dissociation by KCl.

From Table 1 one can also see that the use of 500 mM KCl at the dissociation step and 300 mM KCl in the sucrose gradient was just as effective as using 500 mM KCl in both cases. The advantage of using lower salt in the sucrose gradient is to minimize the salt effect on the subunits which may affect the conformation and activity.

Since nearly 30% of the 80S ribosomes were still resistant to dissociation at high KCl, the question arose

Table 1. Dissociation of run-off ribosomes into subunits under various conditions of incubation

	$MgCl_2$-KCl concentration (mM)		
Polysome incubation conditions	Dissociation step	Sucrose gradients	% Dissociation
EXPT. I			
Control 0°C	5-100	5-100	0
Incubated 37°C	5-100	5-100	4.8
Incubated 37°C + incorporation components*	5-100	5-100	12.1
EXPT. II			
Incubated 37°C	3-300	3-300	25.3
Incubated 37°C	5-500	3-300	32.9
Incubated 37°C	5-500	5-500	33.1
EXPT. III			
Incubated 37°C + incorporation components	3-300	3-300	65.8
Incubated 37°C + incorporation components	5-500	3-300	72.6
Incubated 37°C + incorporation components	5-500	5-500	70.0

*All components required for amino acid incorporation as described in Materials and Methods.

as to whether the supply of energy or supernatant factors were adequate. Table 2 shows the results of an experiment in which the regular amount as well as a supplemental amount of creatine phosphate + creatine phosphokinase and supernatant factors were used. The additional factors increased the percent dissociation of run-off 80S from 63% to 80%. Initially this appears as an improvement, however, if compared with other experiments in which the routine dissociation was 70%, then the difference here is not as significant.

All experiments up to this point had been done using 4 mM Mg^{2+} during the incubation, therefore, the effect of Mg^{2+} concentration on polysome "run-off" was examined. The optimum concentration was found to be in the range of 2.5 to 3.5 mM Mg^{2+} (Table 3). Changing the Mg^{2+} concentration from 4.0 mM to 3.0 mM might improve the dissociation slightly, but this would still not account for the remaining KCl-resistant 80S ribosomes.

The quality of the isolated polysomes seemed to be the most critical factor in determining the extent of dissociation. Using carefully prepared polysomes and fresh supernatant enzymes (i.e. never frozen) total run-off of 80S particles was obtained which would dissociate completely into subunits at 500 mM KCl. However, after scaling up the procedure 50-fold, the best dissociation that could be routinely obtained was 80-90%. This same phenomenon has also been observed before (Falvey, Staehelin, 1970).

Table 2. Effect of additional supernatant factors and creatine phosphate + creatine phosphokinase during incubation on the dissociation of run-off 80S ribosomes into subunits.

	$MgCl_2$-KCl concentration (mM)		
Polysome incubation conditions	Dissociation step	Sucrose gradient	% Dissociation
Regular components*	5-100	5-100	2.8
	5-500	5-500	63.0
Additional components†	5-100	5-100	11.5
	5-500	5-500	80.0

*Incubation with regular amount of incorporation components at 37° for 30 min as described in Materials and Methods.
†Incubation at 37° for 20 min with regular components, then additional pH 5 enzyme and creatine phosphate + creatine phosphokinase was added and the incubation continued to 45 min.

Table 3. Effect of Mg^{2+} concentration during incubation on the dissociation of run-off 80S ribosomes into subunits

Polysome incubation conditions	$MgCl_2$-KCl concentration (mM) Dissociation step	 Sucrose gradient	% Dissociation
Control 0°C	5-100	5-100	2.0
2.5 mM $MgCl_2$	5-100	5-100	3.9
	5-500	5-500	62.5
3.5 mM $MgCl_2$	5-100	5-100	4.2
	5-500	5-500	57.0
5.0 mM $MgCl_2$	5-100	5-100	2.5
	5-500	5-500	43.9

Polysomes were incubated at 37° for 30 min in the complete amino acid incorporating system as described in the Materials and Methods. The control sample was incubated in the presence of 4 mM $MgCl_2$.

The remaining 10-20% 80S ribosomes were difficult to separate from the 60S subunit fraction which ultimately resulted in lower yields of pure 60S subunits. In order to obtain complete dissociation puromycin was used to sensitize the remaining "stuck 80S ribosomes".

(c) Treatment of "KCl-resistant" 80S ribosomes with puromycin

Puromycin, either by itself or in combination with supernatant factors, has been used to make ribosomes sensitive to high salt (Lawford, 1969; Von Der Decken *et al.*, 1970; Mechler, Mach, 1971; Blobel, Sabatini, 1971). In order to find the optimum concentration of puromycin for our system polysomes were incubated in the cell-free incorporation system, treated with various concentrations of puromycin, exposed to 0.5 M KCl and then analyzed in sucrose gradients. Without puromycin treatment approximately 30% of the 80S particles remained undissociated (Fig. 4B). At 0.05 mM puromycin only a trace amount of 80S ribosomes remained (Fig. 4C). At 0.1 mM complete dissociation occurred (Fig. 4D). This concentration of puromycin (0.1 mM) is 10- to 20-fold less than has been used by other investigators.

(d) Large scale preparation of ribosome subunits

For the large scale preparation of ribosome subunits, 600-1000 A_{260} units of mouse liver polysomes were used. Figure 5 shows the sedimentation profile of the subunits obtained after the dissociation procedure. The

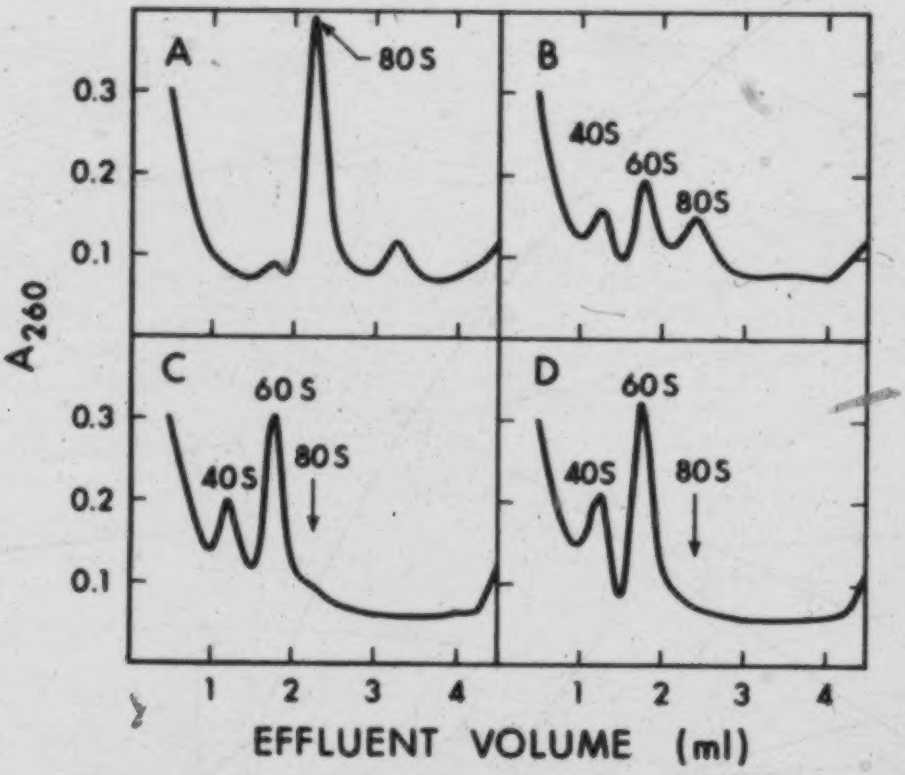


Figure 4. Sucrose density-gradient analysis of "KCl resistant" 80S ribosomes treated with puromycin. Linear sucrose gradients (10-20%) were made up with the same buffer as ribosomes except β-ME, DTE, and glycerol were omitted. Centrifugation was in a Spinco SW 50.1 rotor at 40,000 rpm for 60 min at 4°.

A. Polysomes incubated at 37° for 30 min in the presence of all components required for protein synthesis, analyzed in 5 mM Mg^{2+}-100 mM KCl.
B. Same as A then incubated at 37° for 10 min in 3 mM Mg^{2+}-500 mM KCl, analyzed in 2 mM Mg^{2+}-300 mM KCl
C. Same as A with sample adjusted to 0.05 mM puromycin then incubated at 37° for 10 min in 3 mM Mg^{2+}-500 mM KCl analyzed in 2 mM Mg^{2+}-300 mM KCl.
D. Same as A with sample adjusted to 0.1 mM puromycin then incubated at 37° for 10 min in 3 mM Mg^{2+}-500 mM KCl, analyzed in 2 mM Mg^{2+}-300 mM KCl.

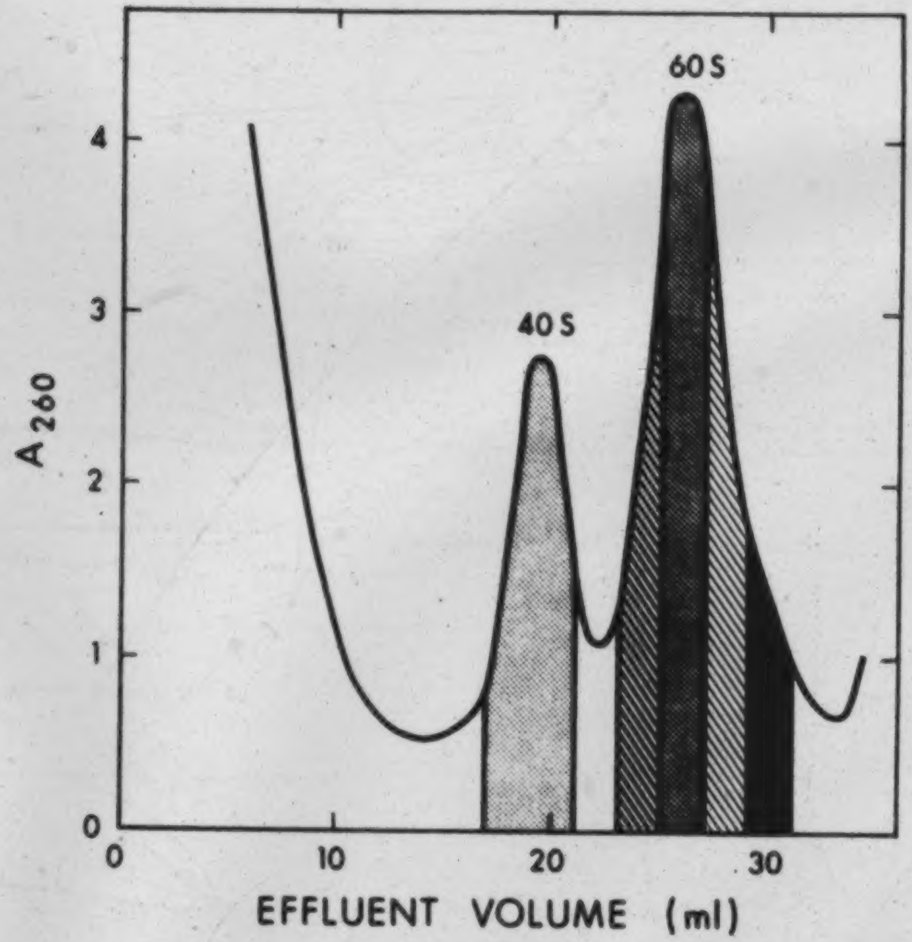


Figure 5. Preparation of mouse liver ribosomal subunits by sucrose density-gradient centrifugation. Reaction mixture for run-off of polysomes was prepared as described in Materials and Methods. 100-150 A_{260} units (5 ml of reaction mixture) were layered onto a 30 ml 0.3-1.0 M convex-exponential sucrose gradient containing 20 mM Tris-HCl (pH 7.4), 2 mM Mg^{2+}, 300 mM KCl, 6 mM β-ME and 0.25 mM DTE. Centrifugation was in a Spinco SW 27 rotor at 25,000 rpm for 14 hr at 4°. The gradients were fractionated (1.9 ml fractions) using a flow cell attached to a Gilford Model 2000 spectrophotometer.

░ 40S

▧ Lead 60S fraction

▓ Center 60S fraction

▨ Late 60S fraction

■ Bottom 60S fraction

40S subunits were collected in one fraction while the 60S were collected as a lead, center, late and bottom fraction.

2. Physical characterization of the isolated ribosome subunits

Four criteria that were used to determine the purity and quality of the isolated ribosome subunits include: (i) a single peak on sucrose density-gradient analysis, (ii) ability of the subunits to reassociate to form 80S ribosomes, (iii) homogeneity of subunit ribosomal RNA and, (iv) measurement of their amino acid incorporating ability. The first three criteria will be described in this chapter while the biological activity is under a separate heading in Chapter III.

(a) Analysis by sucrose density-gradient centrifugation

Figure 6 shows the sedimentation profiles of the subunits when analyzed on linear 10-20% sucrose gradients. The 40S subunits showed no contamination with 60S particles (Fig. 6B) as analyzed at 2 mM Mg^{2+} and 200 mM KCl (K^+/Mg^{2+} ratio 100). When the isolated 40S subunits were analyzed at 1 mM Mg^{2+} - 100 mM KCl (K^+/Mg^{2+} ratio 100), a slight shift of the peak to the heavier side was noticed. This shift was small and was thought to represent a configurational change rather than dimerization. Other research groups have found mouse liver 40S subunits to dimerize

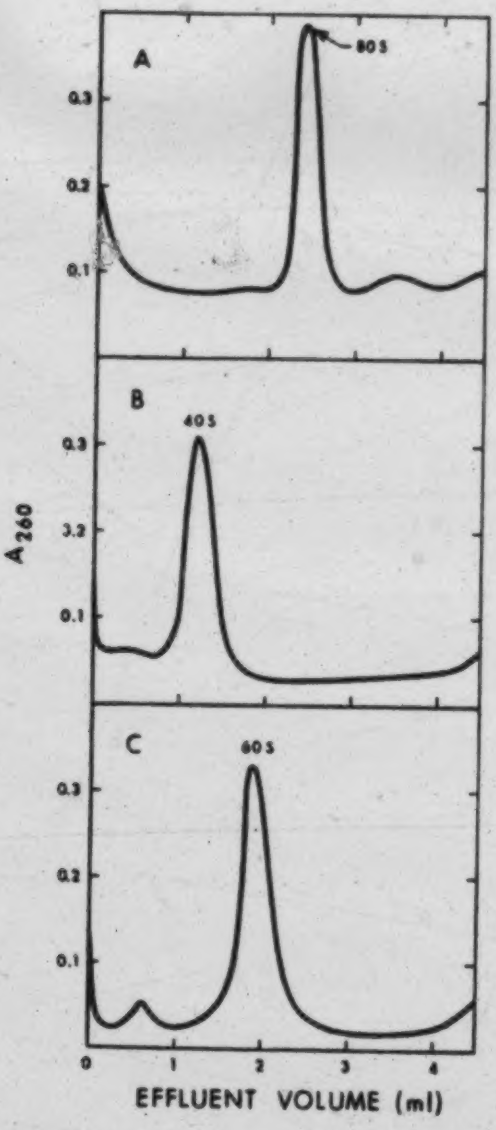


Figure 6. Sucrose density-gradient analysis of isolated ribosome subunits. Linear sucrose gradients (10-20%) were made up in 20 mM Tris-HCl (pH 7.4) buffer containing the indicated amounts of Mg^{2+} and KCl. Approximately 0.5 A_{260} unit of ribosomes or subunits was analyzed. Centrifugation was in a Spinco SW 50.1 rotor at 40,000 rpm for 60 min at 4°.

A. Run-off 80S ribosomes in 5 mM Mg^{2+}-100 mM KCl
B. 40S subunits in 2 mM Mg^{2+}-200 mM KCl
C. 60S subunits in 2 mM Mg^{2+}-100 mM KCl

only when exposed to a much lower K^+/Mg^{2+} ratio. However, this does illustrate that not only the ratio but also the absolute amount of K^+ and Mg^{2+} are important in determining the sedimentation properties of the isolated subunits.

Initial analysis of the 60S subunits showed a small shoulder of heavier sedimenting material (results not shown). This material was removed by resedimenting the 60S subunits on a preparative convex-exponential sucrose gradient containing 5 mM Mg^{2+}, and collecting only the center fraction of the 60S peak. These 60S subunits (Fig. 6C) were free of 40S subunits and more importantly, of 80S ribosomes.

(b) Reassociation of ribosome subunits to form 80S ribosomes

Isolated 40S and 60S subunits were mixed at various ratios at 5 mM Mg^{2+} (at the same total A_{260}) and then analyzed by sucrose gradient centrifugation. In all cases 80S ribosomes were formed with the optimum A_{260} ratio of 40S/60S subunits being approximately 1/2 (Fig. 7). This reassociation was spontaneous and did not require the addition of tRNA, mRNA or supernatant factors. At a higher 40S/60S ratio (i.e. 1/1), the excess 40S appeared to form aggregates showing a broad sedimentation pattern (Fig. 7A).

In experiments described later (Chapter III, Results, section 1) the highest amino acid incorporation was obtained in the presence of 40S and 60S particles in a ratio of 1/2.5 This is nearly equivalent to the theo-

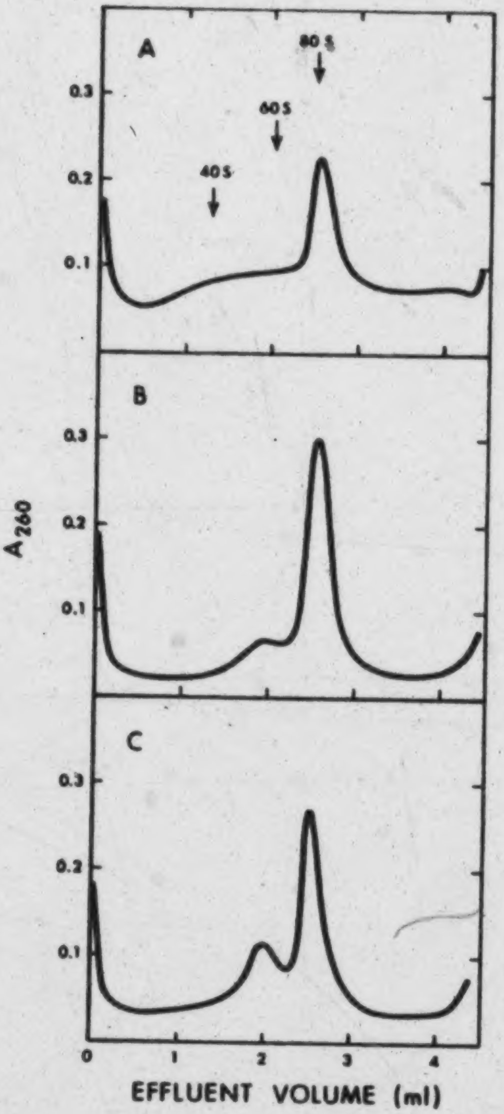


Figure 7. Reassociation of isolated 40S and 60S subunits to form 80S ribosomes. The samples were analyzed as described in Fig. 6 with gradients containing 20 mM Tris-HCl (pH 7.4), 5 mM Mg^{2+} and 100 mM KCl. Ribosome subunits were mixed in the indicated A_{260} ratios, adjusted to 5 mM Mg^{2+} - 100 mM KCl and incubated at 37° for 5 min.

A. 40S/60S A_{260} ratio 1/1
B. 40S/60S A_{260} ratio 1/2
C. 40S/60S A_{260} ratio 1/3

retical ratio[1] of 1/2.4 for the stoichiometric association of 40S and 60S subunits. This indicates that essentially all of the isolated 40S and 60S subunits are structurally intact and are able to form 80S ribosomes.

(c) Analysis of ribosomal RNA

The extent of cross-contamination of the subunit preparations as well as subunit structural integrity can be determined by extracting and analyzing their RNA. The results of such an experiment can be seen in Figure 8. Run-off 80S ribosomes showed 18S and 28S RNA in a ratio of 1/2.4 (Fig. 8A). The 40S subunit preparation gave 18S RNA free from contamination by 28S RNA (Fig. 8B) while the 60S subunits showed 90% 28S RNA with the remainder comprising a 20S component and a low molecular weight RNA which likely represented 5S RNA. No 18S RNA was observed indicating no contamination by 40S or 80S ribosomes. The origin of the 20S RNA was not clear but may be a degradation product of the 28S RNA.

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[1] The theoretical ratio was determined from the molecular weight of the 18S and 28S ribosomal RNA, assuming the 40S subunit contains one molecule of 18S RNA, MW 0.7×10^6 daltons; and the 60S subunit contains one molecule of 28S RNA, MW 1.7×10^6 daltons; 40S/60S $\equiv$ one 18S RNA/one 28S RNA $\equiv 0.7 \times 10^6/1.7 \times 10^6 \equiv A_{260}$ ratio 1/2.4 (Petermann, Pavlovec, 1966; Hamilton, 1967).

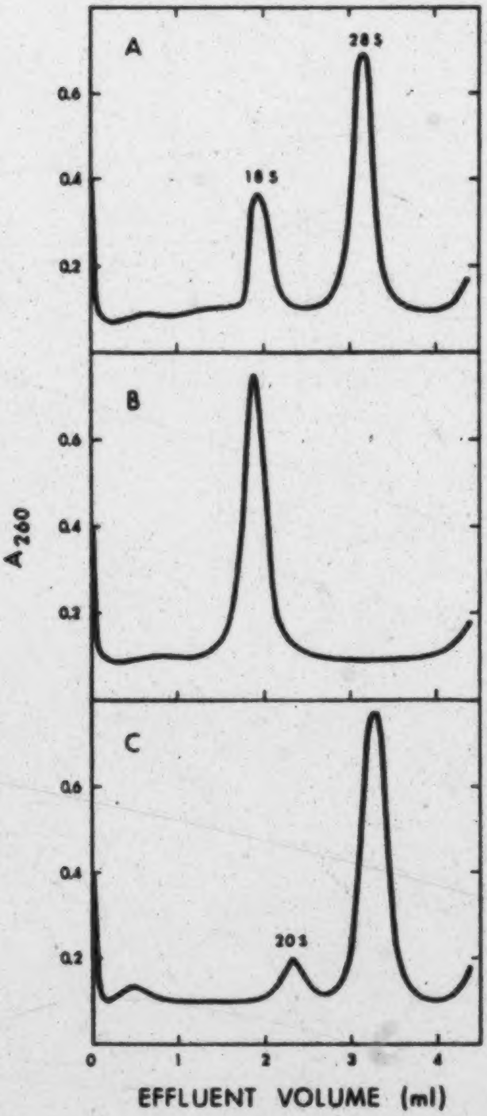


Figure 8. Sucrose density-gradient analysis of ribosomal RNA. Linear sucrose gradients (10-20%) were made up with 20 mM Tris-HCl (pH 7.4), 1 mM Mg^{2+} and 50 mM NaCl. Centrifugation was in a Spinco SW 50.1 rotor at 48,000 rpm for 2.5 hr.

A. RNA extracted from run-off 80S ribosomes
B. RNA extracted from 40S subunits
C. RNA extracted from 60S subunits

(d) Analysis of ribosomal proteins

The ribosomal proteins isolated from the 40S and 60S subunits were analyzed by polyacrylamide gel electrophoresis (Figure 9). A large number of protein bands were detected in each case. Although precise comparisons are difficult, it is clear that the band patterns of the proteins from the 40S and 60S subunits are distinct from each other. A number of bands near the top of the gel of the 80S ribosome are not detected in either the 40S or 60S fraction. These bands may represent loosely-bound proteins such as initiation factors, which were subsequently removed in the process of preparation of the subunits.

A question of current interest is whether ribosomes from various tissues, and also from tissues of different species, contain the same ribosomal proteins. An earlier study on ribosome subunits isolated from L5178Y mouse lymphoma cells (Faber, Tamaoki, 1972) included the polyacrylamide gel analysis of the ribosomal proteins. Comparison of the gel patterns of ribosomal proteins from mouse liver and mouse lymphoma revealed some differences (Fig. 10). The methods used for the isolation of subunits and extraction and analysis of proteins were the same for both tissues. The numbers associated with the protein peaks on the densitometer tracings are purely for ease of comparison and do not represent the actual number of protein bands, since a

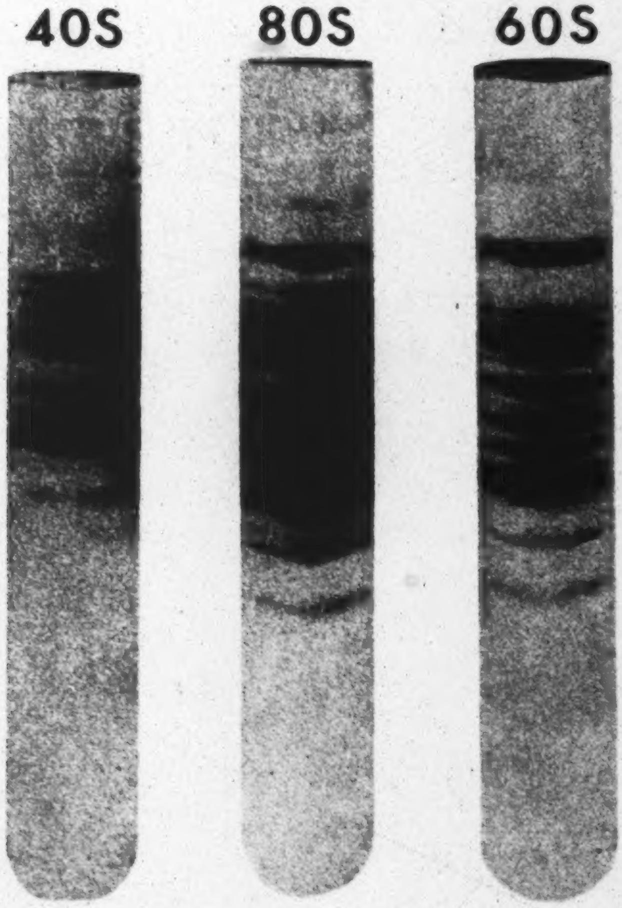


Figure 9. Analysis of mouse liver ribosomal proteins by polyacrylamide gel electrophoresis. Ribosomal proteins were extracted as described in Materials and Methods from run-off 80S ribosomes, 40S and 60S ribosome subunits. Electrophoresis was carried out in 10% polyacrylamide gels containing 6 M urea at pH 4.5 with migration from the anode to cathode at 4 mA/gel. The proteins were stained with 1% Amido Black in 7.5% acetic acid for 1 hr and then destained electrophoretically.

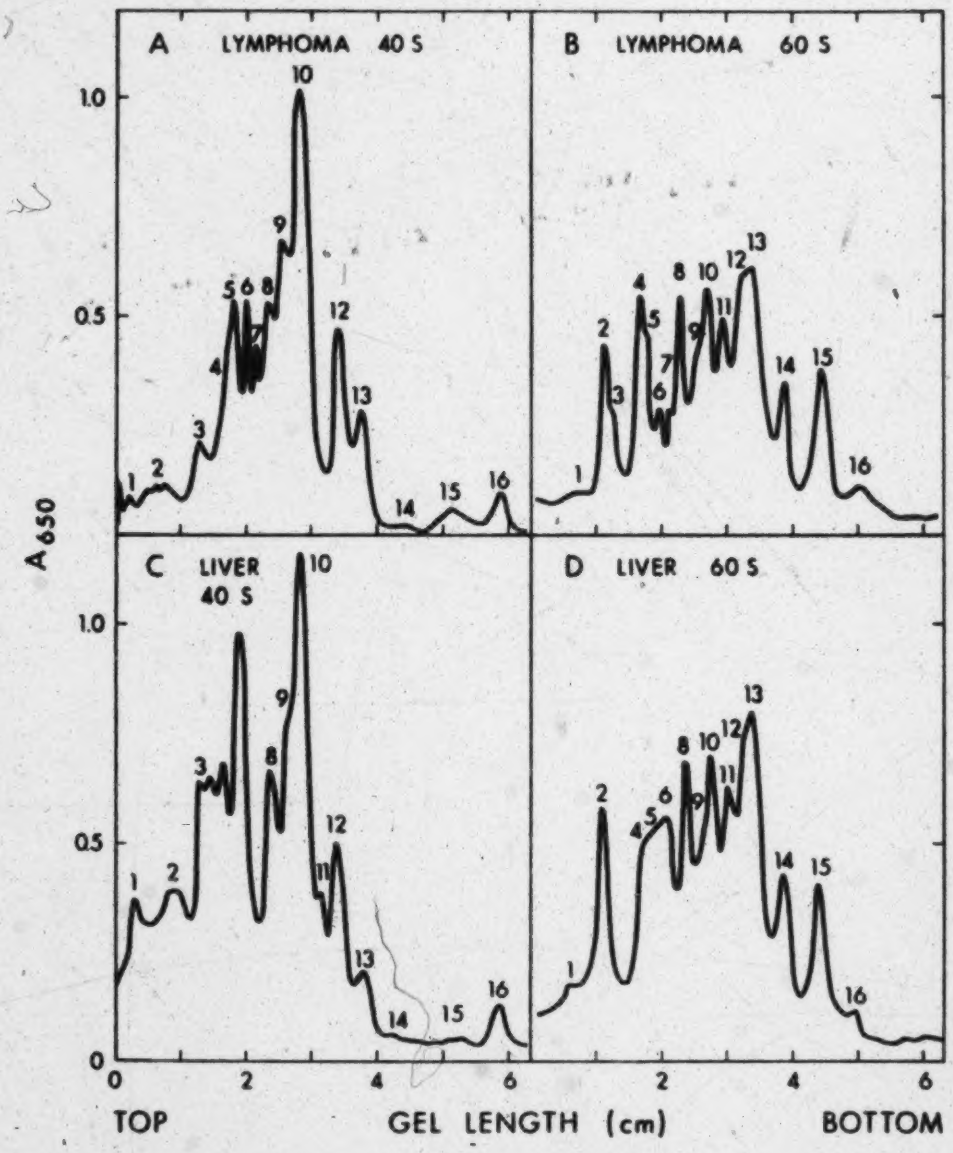


Figure 10. Comparison of mouse liver and L5178Y mouse lymphoma ribosomal proteins. Polyacrylamide gel electrophoresis was carried out as described in Figure 9. Gels were scanned at A_{650} using a Gilford Linear Transport apparatus attached to a Gilford Model 2000 spectrophotometer.

A. Lymphoma 40S ribosomal proteins
B. Lymphoma 60S ribosomal proteins
C. Liver 40S ribosomal proteins
D. Liver 60S ribosomal proteins

single band may contain several different proteins (Fogel, Sypherd, 1968; Hardy *et al.*, 1969).

The ribosomal protein patterns for the 60S subunits of lymphoma and liver cells were nearly identical (Fig. 10B and 10D). Peaks 1 through 16 correspond in mobility as well as in peak height with the only area of difference being between peaks 4 to 6. Proteins from the lymphoma and liver 40S subunits, on the other hand, show substantial differences in the slower migrating proteins (peaks 1-7) although proteins 8-16 were essentially the same (Fig. 10A and 10C).

D. Discussion

Early attempts to dissociate ribosomes by using magnesium chelating agents, such as ethylene diamine tetraacetic acid (EDTA) (Lamfrom, Glowacki, 1962; Tashiro, Morimoto, 1966) resulted in subunits with poor biological activity. Martin and Wool (1968) successfully used high concentrations of KCl to obtain ribosome subunits that were active in poly(U)-directed Phe incorporation. Since that time, other methods have become available in which treatment with high concentrations of KCl are not necessary; preincubation of polysomes (Falvey, Staehelin, 1970) and the use of puromycin (Lawford, 1969; Blobel, Sabatini, 1971; Von Der Decken *et al.*, 1970; Mechler, 1971) being two other methods.

For the analysis of 80S ribosomes and subunits, linear sucrose gradients were found to be adequate. The practical limitation of this type of gradient is that particles slow down as they pass through the gradient which results in a loss of resolving power. In 1967 Noll published a method for making an isokinetic gradient in which all particles with the same density sediment at a constant rate throughout the gradient. The advantage of this method is that with increasing time, separation between particle peaks increases allowing high resolution of complex patterns. These gradients therefore can be used as a diagnostic test for the quality of a polysome preparation. In our preparations 9-11 separate bands were observed. The range of S-values for the polysomes was estimated to be 80-450S with an average value of 360S (Noll, 1967).

The importance of the optimum K^+/Mg^{2+} ratio in dissociating ribosomes has been noted previously (Faber, Tamaoki,1972). Too low a value results in poor dissociation while too high a value results in breakdown of the ribosome particle. Disintegration of the ribosome particle is believed due to loss of stabilizing components, primarily Mg^{2+} ions (Chao, Schachman, 1956; Tissieres, Watson, 1958; Hamilton, Petermann, 1959). Mg^{2+} ions are partially responsible for the forces that bind the ribosomal components together. Traub and

Nomura (1969), in studies on the reassembly of the components of *E. coli* 30S subunits, have found that Mg^{2+} and K^{+} ions were absolutely required for the correct placement of the ribosomal proteins on the 16S ribosomal RNA molecule. These cations are believed to neutralize the negative charges of the phosphodiester linkages in the RNA molecule (Watson, 1964). This would allow nonionic weak bonds such as hydrogen bonds and hydrophobic interactions to associate the ribosomal proteins with the ribosomal RNA. A second role for Mg^{2+} ions involves the binding of the small subunit to the large ribosome subunit. In this case the divalent cations neutralize the anionic charges between the "contacting surfaces" of the subunits thereby allowing other interactions to occur which give a high degree of orientation and alignment to the subunit reassociation. There is evidence for both protein-protein interactions (Morgan *et al.*, 1963; Tamaoki, Miyazawa, 1967; Lerman *et al.*, 1966) as well as "Watson-Crick" type RNA-RNA interactions (Marcott-Queiroz, Monier, 1965; Moore, 1966) between the subunits. Therefore the optimum K^{+}/Mg^{2+} ratio for dissociation would appear to displace sufficient Mg^{2+} ions to cause separation of the ribosome into subunits while insufficient to cause changes in the ribosomal RNA-protein interaction within the subunits.

The optimum concentration of puromycin for our system was one-tenth that normally used by others (Lawford, 1969; Blobel, Sabatini, 1971) but was sufficient to bring about complete dissociation of ribosomes because of the relatively small amount of "stuck 80S ribosomes" in the polysome preparations employed. Under these conditions there will be fewer peptidyl-puromycin derivatives formed which may be capable of sticking to ribosomes. Subunits prepared in this way should, therefore, be cleaner and possibly more active.

The explanation for the observed slight shift in sedimentation profile of the isolated 40S subunits could involve a change in conformation to a more compact structure. This change was reversible by increasing the KCl concentration from 100 mM to 200 mM at a constant K^+/Mg^{2+} ratio of 100. Since increased salt was required to obtain the original conformation, this suggests that some internal anionic groups are not properly neutralized. Animal ribosomes are known to contain the polyamines, spermine, spermidine, cadaverine and putresine (Zillig *et al.*, 1959). These polyamines have been shown to stabilize ribosomes against enzymatic and thermal degradation and to prevent dissociation in low Mg^{2+} buffers (Siekevitz, Palade, 1962; Ohtaka, Uchida, 1963). The exposure to 0.5 M KCl in the process of preparation of 40S subunits may have displaced some of the bound polyamines causing the change in conformation.

An alternative explanation would be that the subunits have dimerized, although for most 40S subunits a K^+/Mg^{2+} ratio much lower than 100 was required (Lawford, 1969; Petermann, 1971). In an electron microscopy study, Nonamura, Blobel and Sabatini (1971) found that a K^+/Mg^{2+} ratio of approximately 5 (25 mM K^+/ 5 mM Mg^{2+}) was required to dimerize their isolated rat liver 40S subunits. Falvey and Staehelin (1970) prepared both mouse and rat liver ribosome subunits and found the mouse liver 40S subunits to be less susceptible to dimerization than the rat liver 40S subunits. These observations make it unlikely that our 40S subunits had dimerized under the conditions used.

Rat liver 60S subunits have been shown to form dimers at 4°C in low K^+ buffers (Martin *et al.*, 1969). Our isolated mouse liver 60S ribosome subunits showed no tendency to change conformation or dimerize under various ionic conditions of gradient analysis.

The isolated mouse liver 40S and 60S subunits were evidently physically intact since they readily reassociated to form 80S ribosomes at ionic concentrations routinely used for isolation and storage of ribosomes (5 mM Mg^{2+}, 100 mM K^+). Cross-contamination of the 40S and 60S subunits was very low as assayed by sucrose gradient analysis of the subunits and extracted ribosomal RNA. The initial 60S subunit fraction contained about 13% 40S subunits which was reduced to 2% by a second gradient separation. The 28S ribosomal RNA extracted from the 60S subunits showed 10% of the total UV-absorbing material to sediment at 20S which may represent a cleavage product. Shakulov *et al* (1962) have shown that breakage of the polyribonucleotide within the ribosome can occur due to mechanical breakage or ribonuclease action, without any changes in the physicochemical properties of the subunit.

The significance of the difference in gel patterns of ribosomal proteins of the 40S subunits of mouse liver and mouse lymphoma are unknown at this time. Some ribosomal proteins are believed to differ from species to species (Otaka *et al.*, 1968; Takagi *et al.*, 1970; Low, Wool, 1967). Mutolo *et al* (1967) have shown by gel electrophoresis that ribosomal proteins from sea urchin embryos differed by as much as 50% from chick liver ribosomal proteins. These differences could not be accounted for by contamination with cytoplasmic protein since examination of tissues of the same embryos or those at different developmental stages showed no difference in ribosomal protein patterns. These results suggest that some ribosomal proteins are species-specific.

Girolamo and Cammarano (1968) undertook a meticulous study of ribosomal proteins isolated from tissues of several animals. Their results also support the concept of some ribosomal proteins being species-specific. The most interesting observation from their work was that only the protein patterns of the small subunit

(32S), isolated from several tissues of the same animal, were different.

Evidence has also been noted for differences in ribosomal proteins of various organs in the same animal (Huynh-Van-Tan, *et al.*, 1971; MacInnes, 1972; Delaunay, 1972). Generally the differences are smaller than those described above. MacInnes (1972) compared the ribosomal protein patterns of the 40S and 60S ribosome subunits of mouse liver and brain. In both the small and the large subunits, he noted consistant quantitative differences in some of their protein components. However, no major protein bands were present in one tissue and entirely absent in the other.

Support for our observation that the differences in the ribosomal protein patterns of the 40S subunits of liver and lymphoma are authentic include: (i) the 80S ribosomal protein patterns of mouse liver and lymphoma were not identical, (ii) the prepared subunits were physically and functionally intact which was not shown in other studies (capable of reassociation to form 80S ribosomes active in poly(U)-directed phenylalanine incorporation), and (iii) the 60S subunit protein patterns were nearly identical in both electrophoretic mobility and peak amplitude, indicating reliability in the method of analysis.

To try to answer why some ribosomal proteins differ among tissues would be very speculative at this time. Several proteins are known to exist which associate with ribosomes transiently. They include initiation, elongation and termination factors which may associate with ribosomes of different species in varying degrees of affinity. Thus differences observed in 80S ribosomal protein patterns from various tissues could be explained by this phenomenon. However, in the case of ribosome subunits most of the transiently bound proteins can be removed during the preparation procedure. The difference, therefore, reflects the more tightly-bound ribosomal protein components.

Since the small subunit participates in the initial step of protein synthesis, it is interesting to speculate that these observed differences might be associated with the regulation of protein synthesis.

Recently Bollen, Petre and Grosjean (1972) have observed the release of three ribosomal proteins (S1, S2, one unassigned species) from *E. coli* 30S subunits that are engaged in the initiation complex. Two are known to function in a supporting role for the initiation of protein synthesis. Protein S1 is required for optimal messenger RNA binding to the ribosome (Van Duin, Kurland, 1970), while protein S2 in combination with other ribosomal proteins stimulates the binding of f-Met-tRNA to 30S ribosomes in the presence of messenger RNA (Randall-

Hazelbauer, Kurland, 1972). These results suggest that "fractional proteins" (i.e. present in amounts less than one copy per 30S subunit) cycle from free 30S subunits to those engaging in protein synthesis. Whether a similar mechanism is functioning during initiation in eucaryotes remains to be determined.

This chapter has dealt with the physical properties of the isolated ribosome subunits. The most critical question to be answered in any purification procedure is whether the isolated component still retains its biological activity. Chapter III describes experiments that show the biological activity of the isolated subunits as well as the properties of an amino acid incorporation system utilizing precharged tRNA.

CHAPTER III

MAMMALIAN CELL-FREE PROTEIN SYNTHESIS USING POLY(U) AS A SYNTHETIC RNA TEMPLATE

A. Introduction

In a cell-free system first developed by Nirenberg and Matthaei (1961), polyuridylic acid was found to function as the template for phenylalanine (Phe) incorporation. This finding provided experimental evidence for the existence of messenger RNA (mRNA). Another significant contribution of the poly(U)-directed Phe incorporation system has been to provide a model whereby ribosomes, supernatant enzymes and other factors can be tested for their ability to support protein synthesis. Ribosome subunits were first implicated in the process of protein synthesis by Gilbert (1963). He found that both the 30S and 50S ribosome subunits of *E. coli* were required for poly(U)-directed [^{14}C]Phe incorporation. Sucrose density-gradient analysis of the reaction mixture showed formation of a ribonuclease sensitive Phe incorporating complex which had a range of sedimentation coefficients from 140-200S. The conclusion from these experiments was that several 70S ribosomes had attached to poly(U) in the process of translating the message.

Conclusive evidence for the involvement of the 30S subunit rather than the 70S ribosome in the initiation process came from work done by Nomura and coworkers (Nomura, Lowry, 1967; Nomura, Lowry, Guthrie, 1967; Guthrie, Nomura, 1968). Their results indicated N-formylmethionyl-$tRNA_F$ bound preferentially to 30S subunits programmed by poly(AUG) or f_2 RNA and following the addition of the 50S subunit, formed an active protein-synthesizing complex.

The preparation of active mammalian ribosome subunits has been much more difficult than from procaryotes. The first methods to produce ribosome subunits used pyrophosphate, EDTA or low Mg^{2+} concentration (Lamfrom, Glowacki, 1962; Petermann, Pavlovec, 1969). These subunits characteristically had poor biological activity and in many cases had undergone irreversible structural changes. Martin and Wool (1968, 1969) successfully prepared active mammalian ribosome subunits. Their method relied on the use of a high concentration of KCl (0.88 M) with an incubation at elevated temperatures. By this method they were able to prepare active ribosome subunits from rat liver, rat muscle, rabbit muscle, and a protozoan (*Tetrahymena pyriformis*) and also showed that the hybrid ribosomes were active. Lower concentrations of KCl were found to be effective if the ribosomes were preincubated under protein synthesizing conditions (Falvey, Staehelin,1970) or in the presence of puromycin (Lawford, 1969).

In 1968 Miller and Schweet reported that reticulocyte ribosomes washed with 0.5 M KCl required the addition of this ribosome wash for the *de novo* synthesis of hemoglobin. If it was not added, only nascent peptide chains

In 1968 Miller and Schweet reported that reticulocyte ribosomes washed with 0.5 M KCl required the addition of this ribosome wash for the *de novo* synthesis of hemoglobin. If it was not added, only peptide chains already initiated were completed. Ribosome wash, when added to a poly(U)-directed Phe incorporating system, lowered the Mg^{2+} optimum from 10 mM to 5 mM Mg^{2+} ("Mg^{2+} shift"). These results suggested that ribosome wash stimulated the formation of an initiation complex.

Using the Mg^{2+} shift assay two factors, IF-M_1 and IF-M_2, were shown to be required for the initiation of polyphenylalanine synthesis directed by poly(U) at low Mg^{2+} concentration (Shafritz *et al.*, 1970; Shafritz, Anderson, 1970; Woodley *et al.*, 1972). This assay has also been used to show initiation factor activity in rat liver (Grummt, Bielka, 1971), chick oviduct (Means *et al.*, 1971) and ascites cell-free systems (Leader *et al.*, 1972).

In this chapter, studies on the enzymatic activity of the isolated mouse liver ribosome subunits and run-off 80S ribosomes are described. Two poly(U)-directed Phe incorporation systems were used. The Nirenberg system, using labeled [^{14}C]Phe with crude supernatant enzyme fractions was used because of its simplicity of design. The second, a precharged tRNA system, using labeled aminoacyl-tRNA and peptide chain elongation factors eliminated components necessary for the acylation of tRNA. The properties of the incorporation systems and the utilization of the precharged tRNA system for the assay of initiation factor activity are described.

B. Materials and Methods

E. coli B and yeast tRNA were purchased from General Biochemicals Co., poly(U) from Miles Laboratories Inc., diethylaminoethyl (DEAE)-cellulose from Whatman, and Sephadex G-100 and Ficoll from Pharmacia. Nitrocellulose membrane filters (0.45 μm) were purchased either from Millipore Filter Corp. (Millipore filters) or from R-B Filters Ltd. (Microfil filters). [^{14}C]Phe (455 mCi/mmole) was purchased from Amersham/Searle; [^{3}H]Phe (6.15 Ci/mmole) from New England Nuclear.

Ribosomes

The preparative procedures for mouse liver run-off 80S ribosomes and subunits are described in Chapter II. Total polysomes and membrane-bound polysomes were prepared according to Blobel and Potter (1967) and washed twice with 0.5 M KCl.

Preparation of enzyme fractions

The aminoacyl-tRNA synthetases and crude elongation factors were prepared by modification of the methods of Moldave (1968) and Arlinghaus *et al.* (1968).

(i) Aminoacyl-tRNA synthetases.

The pH 5 precipitate prepared as described in Chapter II was used as a source of aminoacyl-tRNA synthetase. The pH 5 enzyme fraction was adjusted to pH 7.4, treated with 0.05% protamine sulfate (pH 6.5) for 30 min at 0^{o}C with stirring. This was then centrifuged at 12,000 x g (av) for 10 min. $(NH_4)_2SO_4$ was added to the supernatant and the protein fraction precipitated between 25-70% saturation was collected, dissolved in buffer A containing 15% glycerol and dialyzed against the same buffer.

(ii) Crude elongation factors.

Crude elongation factors were prepared from the supernatant obtained after precipitating the pH 5 enzyme. Protamine sulfate was added to this fraction and the precipitate was removed by centrifugation as described above. To the supernatant $(NH_4)_2SO_4$ was added and the protein fraction precipitated between 25-70% saturation was collected, dissolved in buffer A containing 15% glycerol, and dialyzed against the same buffer.

Mouse liver tRNA

To the post-microsomal supernatant fraction a one-hundredth volume of 20% Macaloid and a one-twentieth volume of 20% potassium acetate (pH 5.4) were added and shaken with an equal volume of water-saturated phenol for 30 min at room temperature. The aqueous layer was separated from the phenol layer by centrifugation at 12,000 x g (av) for 10 min. Two and one half volumes of 95% ethanol was added to the aqueous layer and the sample was kept at -30^{o} for at least three hours. The precipitated RNA was collected by centrifugation, dissolved in water, and the remaining phenol extracted with ether. Potassium acetate (20%) was added to the sample to a final concentration of 1% and the RNA precipitated with ethanol as above. The precipitate was dissolved in 2 M LiCl, 0.1 M potassium acetate (pH 5.0). Undissolved material (high molecular weight RNA) was removed by centrifugation. Transfer RNA in the supernatant was precipitated with ethanol and then dissolved in 2 M Tris-HCl (pH 8.5). Macaloid was added to a final concentration of 0.04% and the sample was incubated at $37^{o}C$ for 40 min. Potassium acetate and ethanol were added as above and the precipitated tRNA washed twice with ethanol then twice with ether, dissolved in 50 mM Tris-HCl (pH 7.4), 5 mM Mg^{2+}, 100 mM KCl, 1 mM DTE, 0.25 mM EDTA and dialyzed against the same buffer. For immediate use the tRNA was stored in small amounts at -30°, but for long storage it was kept in powder form.

"Nirenberg" amino acid incorporation system

The assay system for the incorporation of $[^{14}C]$Phe into acid-insoluble material contained per ml: 1 μmole ATP, 0.3 μmole GTP, 10 μmoles creatine phosphate, 50 μg creatine phosphokinase, 200 μg poly(U), 20 μmole Tris-HCl (pH 7.4), 100 μmole KCl, 6 μmole β-mercaptoethanol (β-ME), 10 μmole $MgCl_2$, 0.15 μCi $[^{14}C]$Phe (455 mCi/mmole), 0.03 μmole each of 19 other amino acids, 1.25 A_{260} units of ribosomes, 100 μg mouse liver tRNA, 250 μg crude elongation factor, and 200 μg aminoacyl-tRNA synthetase protein. The 0.2 ml reaction mixtures were incubated at 37° for 30 min and the reaction stopped by the addition of trichloroacetic acid (TCA) to a final concentration of 10%. The samples were placed in boiling water for 10 min, cooled in ice, filtered through 0.45 μm Millipore filters, and washed with 10 ml of 5% TCA. After drying the filters for 10 min under a heat lamp, hot TCA-insoluble radioactivity was measured in toluene-based scintillation fluid using a Nuclear Chicago liquid-scintillation counter.

Acylation of tRNA

Aminoacyl-tRNA synthetases were prepared from *E. coli* and mouse liver as described previously. The reaction mixture for charging of tRNA contained per ml: 0.6 µmole CTP, 4 µmole ATP, 10 µmole creatine phosphate, 50 µg creatine phosphokinase, 50 µmole Tris-HCl (pH 7.4), 10 µmole $MgCl_2$,100 µmole KCl, 6 µmole β-ME, 2-4 mg aminoacyl-tRNA synthetases, 2.5 mg mouse liver tRNA or 5 mg of either yeast or *E. coli* B tRNA, and 20 µCi [^{3}H]Phe or 5 µCi [^{14}C]Phe. The reaction mixture was incubated at 37° for 10 min and a one-tenth volume of 20% potassium acetate (pH 5.4) was added. Transfer RNA was extracted by the H_2O-saturated phenol method as previously described. The first aqueous layer was reextracted twice with phenol. RNA in the final aqueous layer was precipitated by the addition of a one-tenth volume of 1 M NaCl, two volumes of 95% ethanol and $MgCl_2$ to give a final concentration of 10 mM. After 2 hours at -30° , the precipitate was collected by centrifugation at 12,000 x g (av) for 10 min. The pellet was washed three times, initially with 6 ml of 95% ethanol (at -30°), then ethanol/ether (1/1 ratio) and finally ether. The acylated tRNA was dissolved in buffer containing 50 mM potassium acetate (pH 5.4) and 5 mM $MgCl_2$. Excess ether was removed by gently blowing nitrogen gas over the surface. The solution was dialyzed overnight against the same buffer and stored in small amounts (0.05-0.1 ml) at -30°C.

Preparation of peptide chain elongation factors EF_1 and EF_2

The elongation factors EF_1 and EF_2 were prepared from the crude elongation factor preparation by the method of Shafritz and Anderson (1970) with a few modifications. Three ml of crude elongation factor preparation (64 mg protein/ml) was dialyzed overnight against 20 mM Tris-HCl (pH 7.4) containing 1 mM Mg^{2+}, 100 mM KCl, 1 mM DTE, 0.1 mM EDTA, and 15% glycerol. This was placed on a 1.5 x 80 cm Sephadex G-100 column equilibrated with the above buffer, and eluted with the same buffer. The column fractions were assayed for elongation factor activity as described below.

(1) Assay of EF_1 activity

The binding of $[^3H]$Phe-tRNA to run-off 80S or twice-washed ribosomes was assayed using the Millipore filter technique described by Nirenberg and Leder (1964) and as modified by McKeehan and Hardesty (1969). A reaction mixture of 100 µl contained the following components: 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 100 mM KCl, 6 mM β-ME, 0.25 mM DTE, 1 mM GTP, 25 µg poly(U), 0.5 A_{260} unit of ribosomes, 3.0 pmoles of $[^3H]$Phe-tRNA *E. coli* B and 10 µl of each of the fractions being tested. The samples were incubated at 23° for 5 min and the reaction stopped by adding 3 ml of ice cold buffer containing 20 mM Tris-HCl (pH 7.4), 5 mM Mg^{2+} and 100 mM KCl. The samples were filtered through 0.45 µm Microfil membranes and washed three times with 3 ml portions of buffer. The membranes were dried and counted in a liquid scintillation spectrophotometer.

(2) Assay of EF_2 activity

The EF_2 activity of the various column fractions was assayed by measuring the extent of poly(U)-directed polymerization of $[^3H]$Phe in the presence of added EF_1 factor. The full assay conditions are described below.

Precharged tRNA amino acid incorporation system

The polymerization of $[^3H]$Phe or $[^{14}C]$Phe from precharged tRNA was assayed in a 100 µl reaction mixture which contained the following: 20 mM Tris-HCl (pH 7.4), 100 mM KCl, 6 mM β-ME, 0.25 mM DTE, 1 mM ATP, 0.3 mM GTP, 10 mM creatine phosphate, 5 µg creatine phosphokinase, 25 µg poly(U), 50 µg crude elongation factors or 35 µg EF_1 and 40 µg EF_2, 0.2 A_{260} unit of run-off 80S ribosomes, and the amount of $[^{14}C]$Phe-tRNA or $[^3H]$Phe-tRNA and $MgCl_2$ as indicated in the figure legends. The samples were incubated at 37^o for the times indicated in the legends and the reaction stopped by the addition of 1 ml of 10% trichloroacetic acid (TCA). Hot TCA-insoluble radioactivity was measured as described previously.

Preparation of liver ribosome wash

Ribosome wash was prepared by a modification of the methods of Miller and Schweet (1968) and Prichard *et al.* (1971). Livers were homogenized as described in Chapter II for polysome preparation, except that the buffer used contained 25 mM KCl and homogenization was done in 4.5 ml of buffer per gram of liver. The top two-thirds of the post-mitochondrial supernatant obtained by low speed centrifugation (12,000 x g (av) for 10 min) was centrifuged in a Spinco Type 60 Ti rotor at 233,000 x g (av) for 90 min. The post-microsomal supernatant was discarded and the pellets washed several times with standard sucrose buffer (0.25 M sucrose - 1 mM DTE - 0.1 mM EDTA, pH 7.0) and resuspended in the same buffer at 300 A_{260} units/ml. The suspension was adjusted to 0.5 M KCl by the addition of 4 M KCl and stirred at 0° for 30 min. This 0.5 M KCl suspension was centrifuged in a Spinco Type 65 rotor at 175,000 x g (av) for 2 hours. The upper two-thirds of the supernatant was dialyzed overnight against buffer A containing 20% glycerol and stored in small amounts in liquid nitrogen.

DEAE-cellulose chromatography of ribosome wash

The crude 0.5 M KCl ribosome wash was dialyzed for 5 hours against buffer I (10 mM Tris-HCl (pH 7.4), 1 mM Mg^{2+}, 100 mM KCl, 0.1 mM EDTA and 1 mM DTE) followed by a further 2 hour dialysis against the same buffer containing 50 mM

KCl (buffer II). The ribosome wash (10-15 ml, 9 mg protein/ml) was placed on a DEAE-cellulose column (1.5 x 25 cm) previously equilibrated with buffer II. The column was eluted with buffer II and then with a 0.05 M to 0.4 M KCl linear gradient (300 ml). Approximately 3 ml fractions were collected at a flow rate of 30 ml/hour. Samples were taken for the measurement of absorbance at 280 nm and for the determination of the KCl concentration by conductivity meter. The column effluent was divided into several fractions, concentrated 3- to 5-fold, dialyzed against buffer A containing 20% glycerol and stored frozen in small amounts in liquid nitrogen. The ability of each fraction or combination of fractions to stimulate Phe incorporation at 3.5 mM Mg^{2+} was tested using the precharged tRNA system.

C. Results

1. "Nirenberg system" of cell-free amino acid incorporation

(a) Optimum concentration of incorporation components

In the first cell-free incorporation system, the aminoacyl-tRNA synthetases and peptide chain elongation factors were supplied by a crude 105,000 x g (av) cell supernatant. These two enzymatic activities were separated by a pH 5.2 treatment and $(NH_4)_2SO_4$ fractionation and assayed at 5 mM and 10 mM Mg^{2+} (Fig. 11). The optimum level of synthetase at 10 mM Mg^{2+} was 15 μg protein. Higher concentrations of synthetase resulted in a decline of incorporation. The crude elongation factors were required at 100 μg protein to saturate the system, although 50 μg gave an incorporation level equivalent to 92% of the saturating level. In both cases, the incorporation at 10 mM Mg^{2+} was 3- to 7-fold higher than at 5 mM Mg^{2+}. The synthesis of polyphenylalanine was found to be highest at 9 mM Mg^{2+} (Fig. 12A) and proceeded at a linearly up to 0.6 A_{260} unit of 80S ribosomes (Fig. 12B).

(b) Amino Acid incorporation by the isolated ribosome subunits

Table 4 shows the ability of the isolated subunits to incorporate [^{14}C]Phe in the presence of poly(U). In experiment I the 40S subunits were isolated from the gradient in a single fraction while the 60S were collected in four fractions (Chapter II, Fig. 5). The 40S subunits were found to polymerize [^{14}C]Phe less than 1% of that of the 80S control. This agrees with previous results (Chapter II) which indicated the 40S subunits to be essentially free of contamination. The 60S subunit fractions on the other hand, exhibited activity varying from 9.4% to 17.4% of the 80S control. Recombination of the 40S and center 60S subunits in an A_{260} ratio of 1/1.5, produced 80S ribosomes which were as active as the control (Table 4, Expt. I, last line).

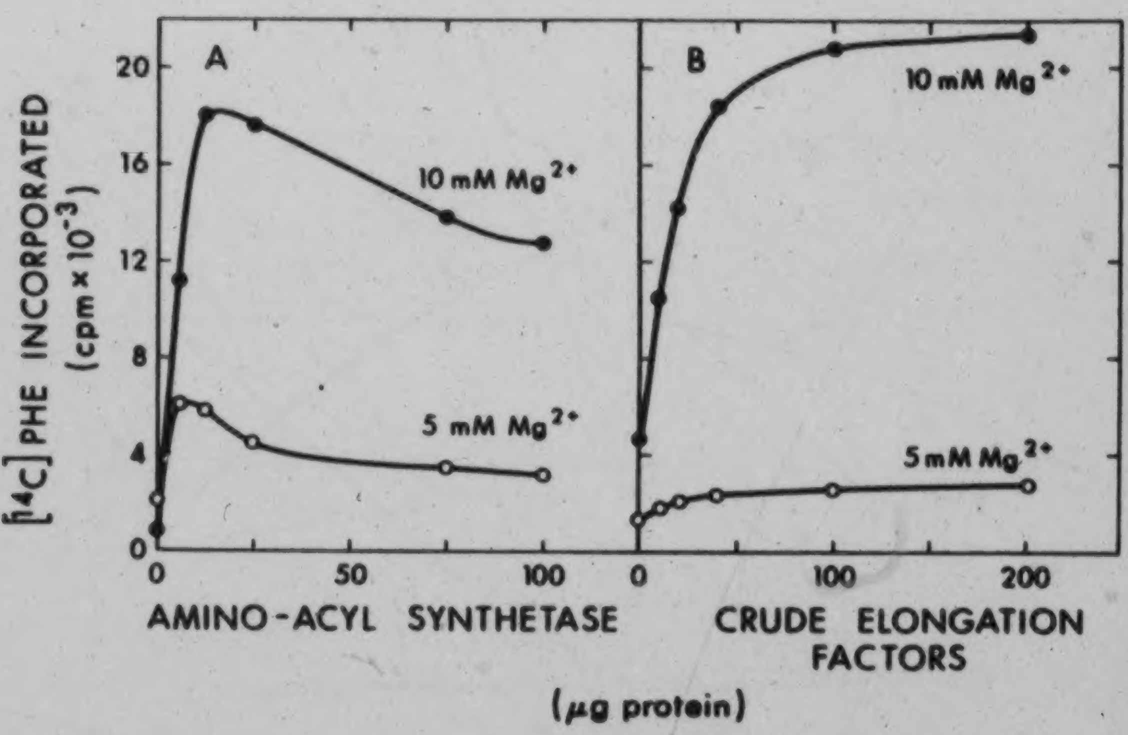


Figure 11. [^{14}C]Phe incorporation as a function of aminoacyl-tRNA synthetase and crude elongation factor concentration. The incorporation was carried out at 37° for 30 min as described in Materials and Methods using 0.5 A_{260} run-off 80S ribosomes. Background count at 0 time (700 cpm) was subtracted.

○——○, 5 mM Mg^{2+}

●——●, 10 mM Mg^{2+}

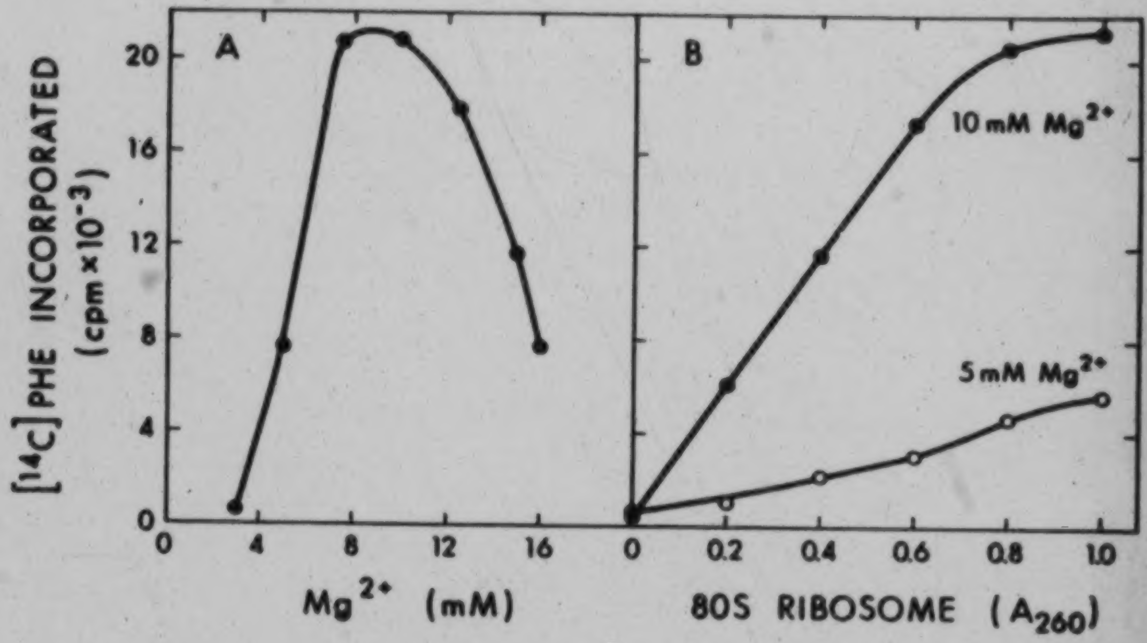


Figure 12. [^{14}C]Phe incorporation as a function of the Mg^{2+} and run-off 80S ribosome concentration . The incorporation was carried out at 37° for 30 min as described in Materials and Methods. For the Mg^{2+} concentration curve (A), 0.5 A_{260} unit of run-off ribosomes, 10 μg aminoacyl-tRNA synthetase and 50 μg crude elongation factors were used. Fig. 12B contained the same components except 25 μg aminoacyl-tRNA synthetase was used. Background count at 0 time (700 cpm) was subtracted.

Table 4. Poly(U)-directed Phe incorporation by mouse liver ribosomal subunits

Experiment I			Experiment II			
	[^{14}C]Phe incorporated			40S/60S	[^{14}C]Phe incorporated	
Ribosomes	pmoles/A_{260}	%Control	Ribosomes	A_{260} ratio	pmoles/A_{260}	%Control
Run-off 80S	42.4	100.0	Run-off 80S	-	52.8	100.0
40S	0.3	0.7	40S	-	0.3	0.6
60S (lead)	6.2	14.6	60S (pure)	-	0.9	1.7
60S (center)	4.0	9.4	40S + 60S	1/3	56.4	106.8
60S (late)	7.3	17.2	40S + 60S	1/2	56.8	107.5
60S (bottom)	7.4	17.4	40S + 60S	1/1.5	48.4	91.7
40S + 60S (center)*	46.8	110.0	40S + 60S	1/1	43.0	81.4
			40S + 60S	2/1	22.9	43.4

Phe incorporation was carried out at 37° for 30 min at 10 mM Mg^{2+} as described in Materials and Methods. Background counts of 450 cpm (0.7 pmole) and 650 cpm (1.0 pmole) were subtracted from the 40S + 60S ribosome and run-off 80S ribosome samples respectively, before calculation of incorporation/A_{260} ribosome. *40S/60S A_{260} ratio of 1/1.5; 1 pmole [^{14}C]Phe was equivalent to 625 cpm.

The 60S subunits from experiment I were further purified by recentrifugation through a convex sucrose gradient containing 5 mM Mg^{2+}. Under these conditions, any 40S subunit present would be removed by coupling with a 60S subunit to form an 80S ribosome. 60S subunits thus purified showed an activity corresponding to less than 2% of that of the run-off 80S ribosomes (Expt. II). The optimum A_{260} ratio of 40S/60S for the maximum activity was found to be about 1/2, and was usually equal to or better than the activity of the control run-off 80S ribosomes. As the ratio increased to 1/1 and 2/1, the incorporating activity decreased to 81% and 43% of the control, respectively. Figure 13 shows the relationship between [^{14}C]Phe incorporation and various A_{260} ratios of 40S/60S subunits. The ratio giving the highest activity was found to be 1/2.5.

The kinetics of Phe incorporation was examined for both the run-off 80S and 40S + 60S ribosomes in the presence and absence of poly(U) (Fig. 14). The results were similar with incorporation being linear for 30 min and strictly dependent on added poly(U). In the absence of poly(U), the 40S + 60S ribosomes had an endogenous activity one-half that of the 80S ribosomes. It appears therefore, that sedimentation of the subunits through the 0.3 M KCl preparative sucrose gradient had the beneficial effect of reducing the endogenous activity.

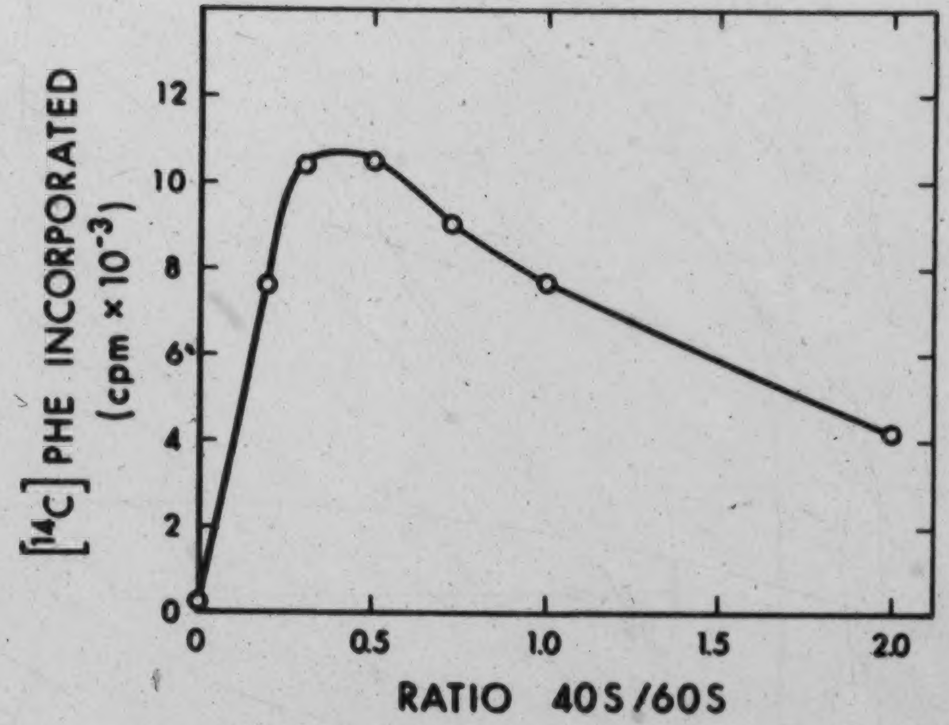


Figure 13. Poly(U)-directed Phe incorporation by 40S and 60S ribosome subunits recombined at various A_{260} ratios. The incorporation was carried out at 37° for 30 min in the presence of 10 mM Mg^{2+} and 0.25 A_{260} 40S + 60S ribosomes as described in Materials and Methods. Background counts at 0 time (450 cpm) were subtracted.

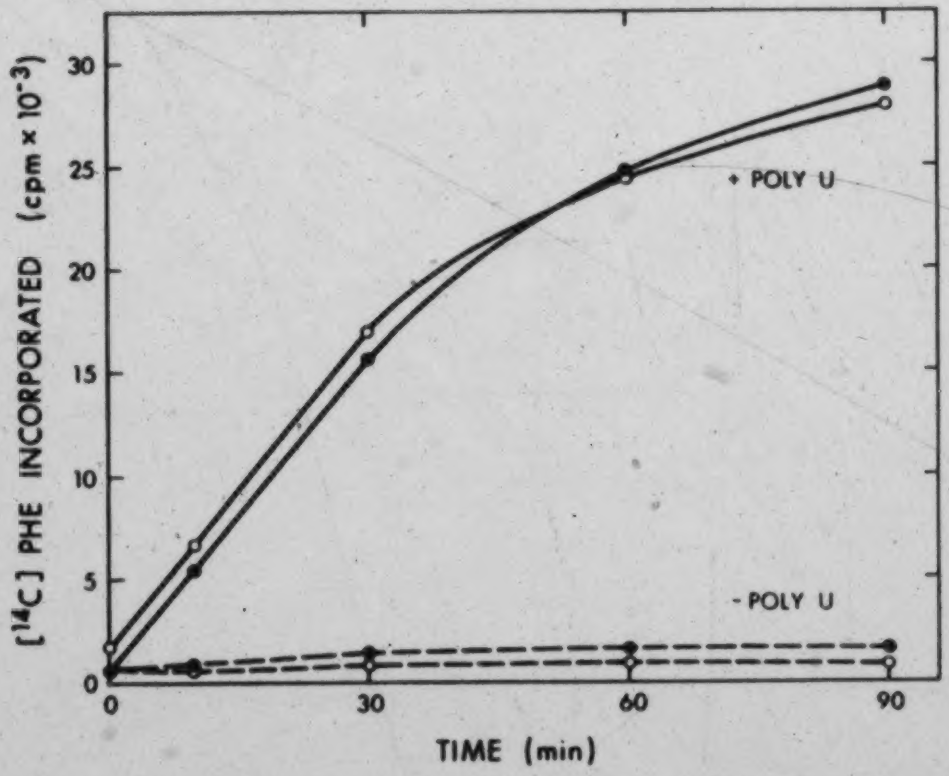


Figure 14. Time course of $[^{14}C]$Phe incorporation by run-off 80S and 40S + 60S ribosomes. The assay was carried out at 37° in the presence of 10 mM Mg^{2+} and 0.25 A_{260} ribosomes. At the indicated times, equal portions of the reaction mixture were removed and hot TCA-insoluble radioactivity determined. Ribosome subunits were present in a 40S/60S A_{260} ratio of 1/2.5. Background counts were not subtracted.

●——●, run-off 80S ribosomes

○——○, 40S + 60S ribosomes

Under the optimum conditions of incorporation (Fig. 14) 185 pmoles of the Phe were incorporated per A_{260} of 40S + 60S ribosomes. Using a molecular weight of 5.0 x 10^6 daltons for the 80S ribosomes (Kuff, Zeigel, 1960; Tashiro, Yphantis, 1965), the number of amino acids polymerized per ribosome can be calculated according to the following equation.

$$\text{Phe polymerized by 80S} = \frac{\text{pmoles Phe incorporated}}{\text{pmoles 80S ribosomes}}$$

$$= \frac{\dfrac{\text{total cpm incorporated}}{\text{cpm/pmole Phe}}}{\dfrac{\text{weight 80S}}{\text{MW 80S}}}$$

Each 40S + 60S couple was found to polymerize on average 9 Phe residues. But it is unlikely that all of the ribosomes present were active in protein synthesis. The number of active ribosomes in an incorporation system can be estimated from the percent that dissociate in a sucrose density-gradient containing 0.3 M KCl. Those ribosomes that are stable in 0.3 M KCl are engaged in polyphenylalanine synthesis (Lawford, 1969; Falvey, Staehelin , 1970). Approximately 60% of our reassociated subunits analyzed after Phe incorporation were found to be active by this criterion. Therefore, each active 40S + 60S couple incorporated 10-15 Phe residues into hot TCA-insoluble polypeptides.

2. "Charged tRNA system" for amino acid incorporation

(a) Phe incorporation using a crude preparation of elongation factors

(i) Incorporation using mouse liver tRNA

A precharged tRNA incorporation system was developed to assay activities of peptide chain elongation and protein initiation factors. A completely homologous mouse liver system was used in the early part of the study since there is evidence both for and against species-specificity of initiation factors (Heywood 1970; Prichard *et al.*, 1971; Metafora *et al.*, 1972; Wigle, Smith, 1973).

Mouse liver [^{14}C]Phe-tRNA supported Phe incorporation linearly up to a concentration of 2 A_{260} units of charged tRNA (Fig. 15). Of the [^{14}C]Phe-tRNA added, 46% was incorporated into hot TCA-insoluble peptides when the incubation was performed at 37°C for 30 min. Higher levels of efficiency (60-70%) were later observed when lower amounts of acylated tRNA was used. This suggests that some other component of the reaction mixture was rate-limiting.

Examination of the kinetics of incorporation showed [^{14}C]Phe to be incorporated at a linear rate for the first 10 min reaching a plateau at 20 min (Fig. 16). In the absence of poly(U), virtually no incorporation took place. Several investigators described similar systems from other mammalian cells which show linear incorporation for 3 to 5 min.

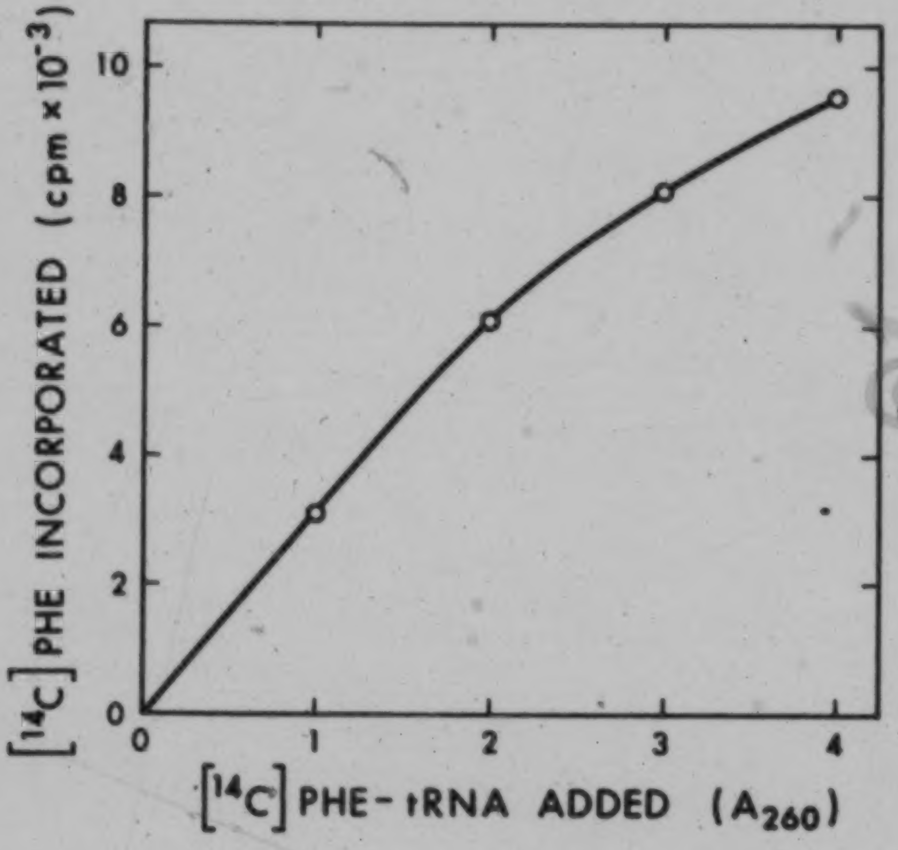


Figure 15. Phe polymerization as a function of mouse liver [^{14}C]Phe-tRNA concentration. The incorporation, in a volume of 200 µl, was performed at 37° for 30 min and contained 0.25 A260 run-off 80S ribosomes, 40 µg poly(U), 50 µg crude elongation factors, 10 mM Mg^{2+} and other components as described in Materials and Methods. Background counts at 0 time (150 cpm) were subtracted. [^{14}C]Phe-tRNA contained 6000 cpm/A_{260} tRNA.

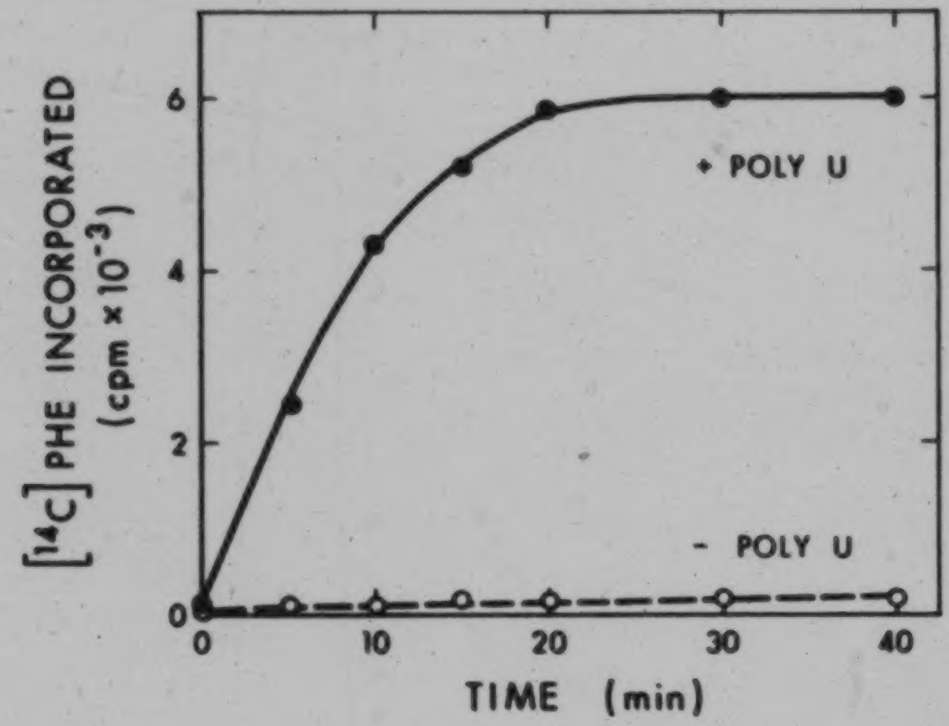


Figure 16. Time course of incorporation using mouse liver [^{14}C]Phe-tRNA as substrate in the presence and absence of poly(U). Conditions of incorporation were the same as Fig. 15. At the indicated times, equal portions of the reaction mixture were removed and hot TCA-insoluble radioactivity determined.

●—●, with poly(U)

○- -○, without poly(U)

(ii) Comparison of Phe incorporation using mouse liver and *E. coli* tRNA

One disadvantage of using mouse liver charged tRNA was the high rate of spontaneous deacylation that occurred upon storage. Within 3-4 weeks approximately 50% of the charged tRNA was deacylated as determined by cold TCA precipitation. *E. coli* charged tRNA was found to be more stable in storage (Marcker, Sanger, 1964), and with its ease of availability offered definite advantages. *E. coli* Phe-tRNA has been shown to serve as substrate in a rabbit reticulocyte cell-free system (Shafritz *et al.*, 1970) and was also found to be capable of supporting polyphenylalanine synthesis in the present cell-free system. Kinetic analysis revealed that liver tRNA supported a faster rate of incorporation than *E. coli* tRNA (Fig. 17). The higher level of incorporation attained by *E. coli* tRNA at 15 min was due to the presence of a slightly larger amount of [^{14}C]Phe-tRNA substrate.

(iii) Stimulation of *E. coli* Phe-tRNA incorporation by mouse liver ribosome wash

Figure 18 shows the stimulation of poly(U)-directed Phe incorporation by the addition of various amounts of liver 0.5 M KCl ribosome wash. The optimal concentration of ribosome wash was 65 μg protein which resulted in a 3.5-fold increase of incorporation over the control

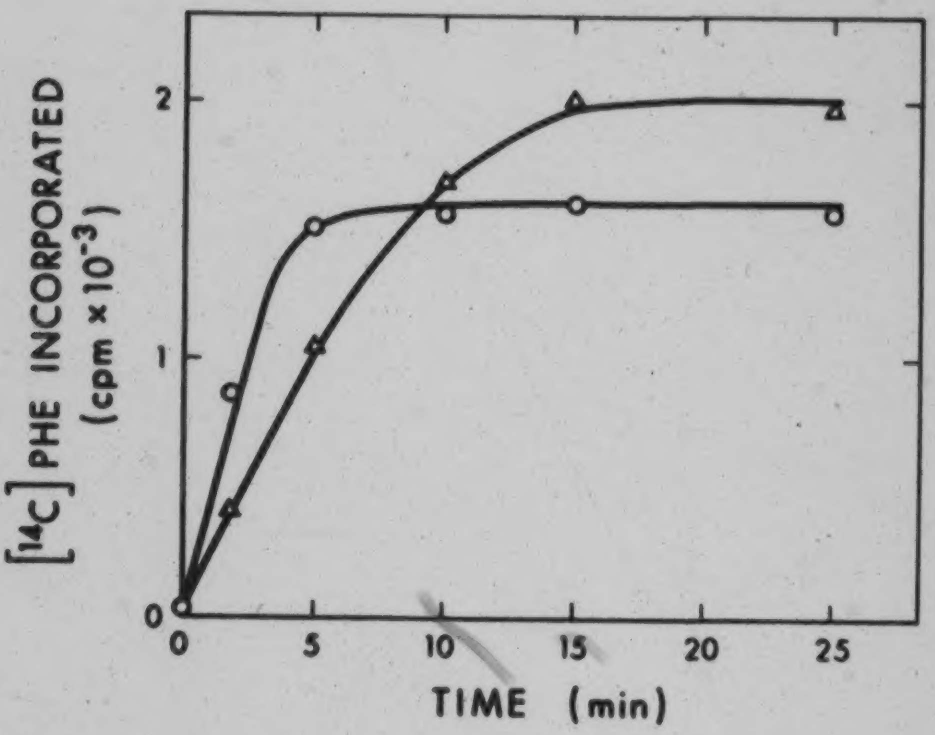


Figure 17. Comparison of the kinetics of Phe polymerization with mouse liver and *E. coli* [^{14}C]Phe-tRNA as substrate. The incorporation was carried out at 37° in the presence of 7.5 mM Mg^{2+}. At the indicated times 100 µl was removed which contained 0.2 A_{260} run-off 80S ribosomes, 25 µg poly(U), 50 µg crude elongation factors, 2800 cpm mouse liver [^{14}C]Phe-tRNA or 3200 cpm *E. coli* [^{14}C]Phe-tRNA, and other components as described in Materials and Methods. Background counts were not subtracted.

○—○, mouse liver [^{14}C]Phe-tRNA

△—△, *E. coli* [^{14}C]Phe-tRNA

(no ribosome wash present). When [^{3}H]Phe incorporation was measured as a function of Mg^{2+} concentration, a "Mg^{2+} shift" from 7.5 to 3.5 mM Mg^{2+} was observed on the addition of ribosome wash (Fig. 19). A time course was also performed at low and high Mg^{2+} in the presence and absence of ribosome wash (Fig. 20). The results again showed a stimulation of incorporation by ribosome wash at low Mg^{2+} concentration.

(b) A more refined incorporation system using partially purified elongation factors, EF_1 and EF_2

(i) Isolation of EF_1 and EF_2

Results described above showed that the precharged tRNA incorporation system was capable of detecting initiation factor activity in the ribosome wash. In order to further characterize the requirements for polypeptide chain initiation and elongation, the elongation factors EF_1 and EF_2 were partially purified. Briefly the method involved a pH 5.2 treatment of the post-ribosomal supernatant, treatment of the soluble fraction with protamine sulfate to remove tRNA, and $(NH_4)_2SO_4$ fractionation and finally resolution of the activities by gel filtration.

Figure 21 shows the separation of EF_1 and EF_2 by Sephadex G-100 chromatography. EF_1 was found to elute shortly after the void volume. This suggests the molecular weight of EF_1 to be less than 150,000 daltons. Addition

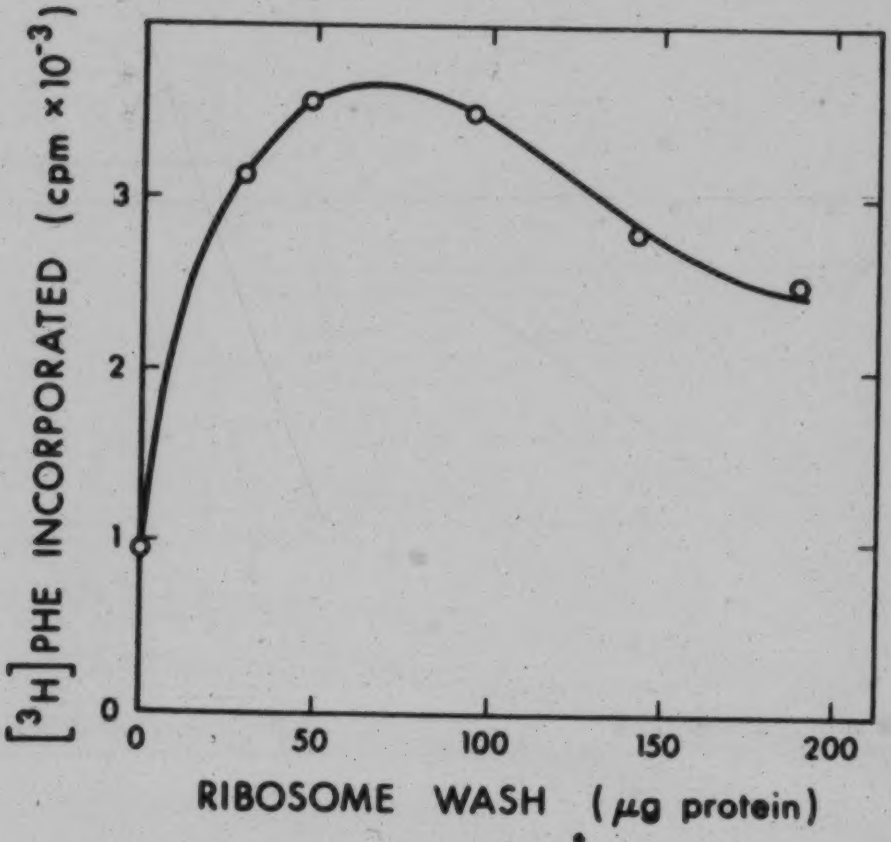


Figure 18. $[^3H]$Phe incorporation as a function of 0.5 M KCl ribosome wash concentration. Incubations, in a total volume of 100 μl, were performed at 37° for 10 min and contained 0.2 A_{260} run-off 80S ribosomes, 50 μg crude elongation factors, 6000 cpm *E. coli* $[^3H]$Phe-tRNA, 3.5 mM Mg^{2+} and other components as described in Materials and Methods. Background counts at 0 time (200 cpm) were subtracted.

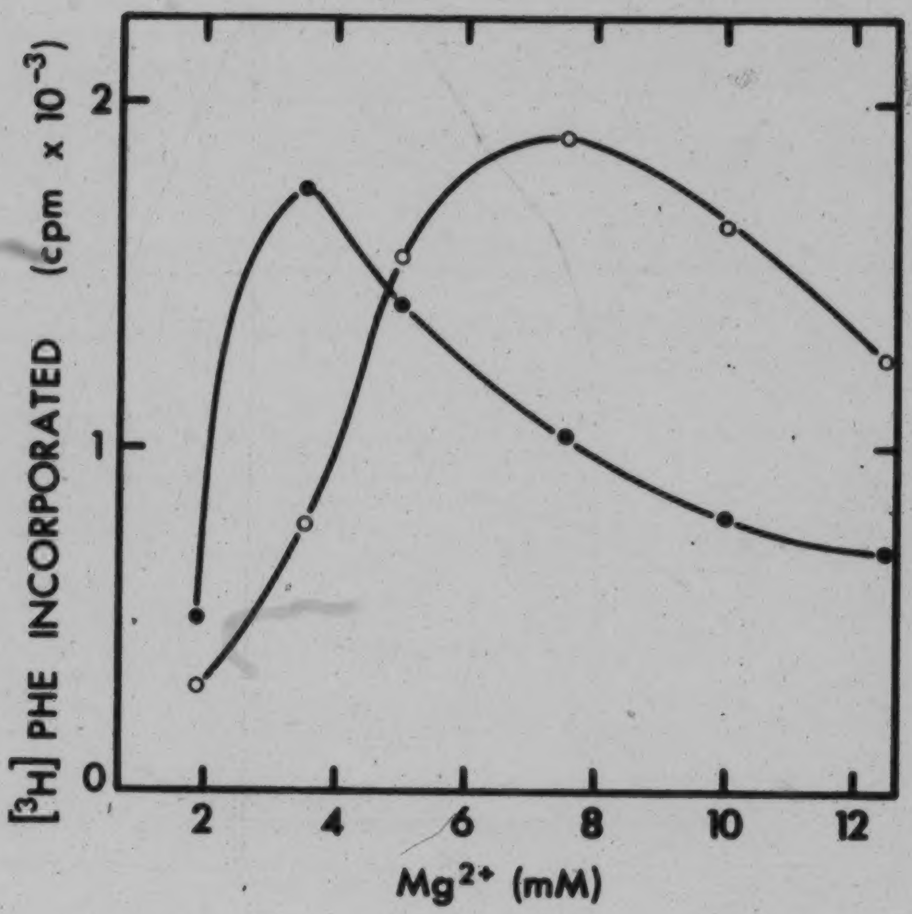


Figure 19. Polymerization of [^{3}H]Phe at various Mg^{2+} concentrations with and without ribosome wash. The incorporation was carried out in a volume of 100 µl at 37° for 10 min and contained 0.2 A_{260} run-off ribosomes, 50 µg crude elongation factors, 90 µg ribosomes wash, 6000 cpm *E. coli* [^{3}H]Phe-tRNA, 3.5 mM Mg^{2+} and other components as described in Materials and Methods. Background counts at 0 time (200 cpm) was subtracted.

●—●, with ribosome wash

○—○, without ribosome wash

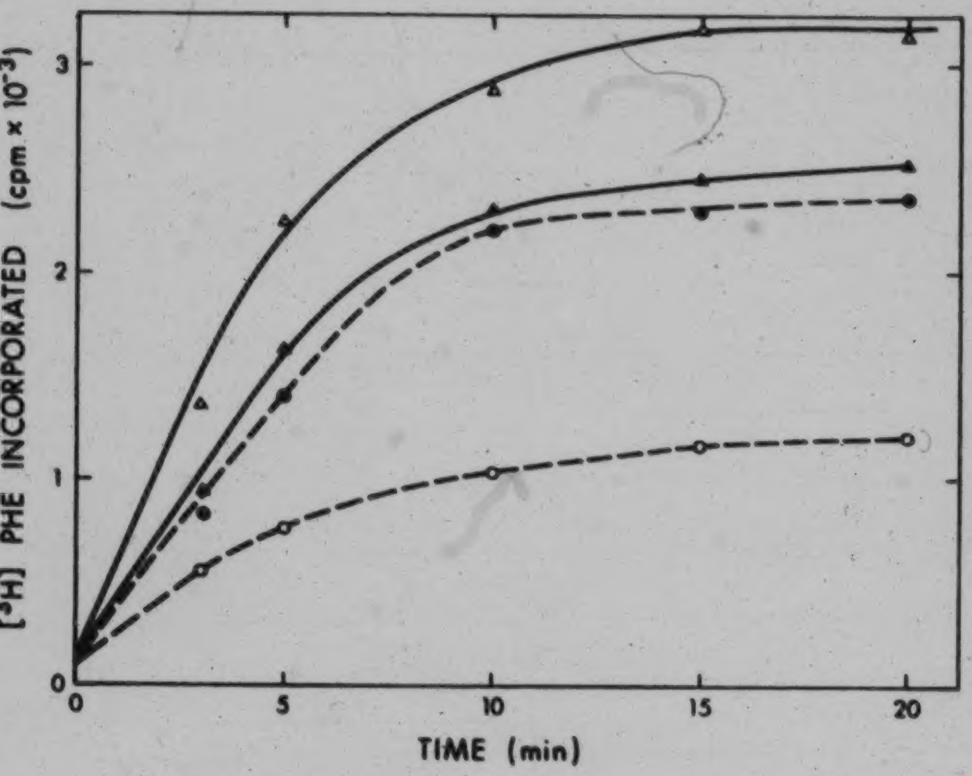


Figure 20. Kinetics of [^{3}H]Phe incorporation at 3.5 mM and 7.5 mM Mg^{2+} in the presence and absence of ribosome wash. Incubation was carried out at 37°. From each reaction mixture, 100 µl was removed at the indicated times, which contained 50 µg crude elongation factors, 90 µg ribosome wash and 6000 cpm *E. coli* [^{3}H]Phe-tRNA and other components as described in Materials and Methods. Background counts were not subtracted.

△ 7.5 mM Mg^{2+}, without ribosome wash
▲ 7.5 mM Mg^{2+}, with ribosome wash
○ 3.5 mM Mg^{2+}, without ribosome wash
● 3.5 mM Mg^{2+}, with ribosome wash

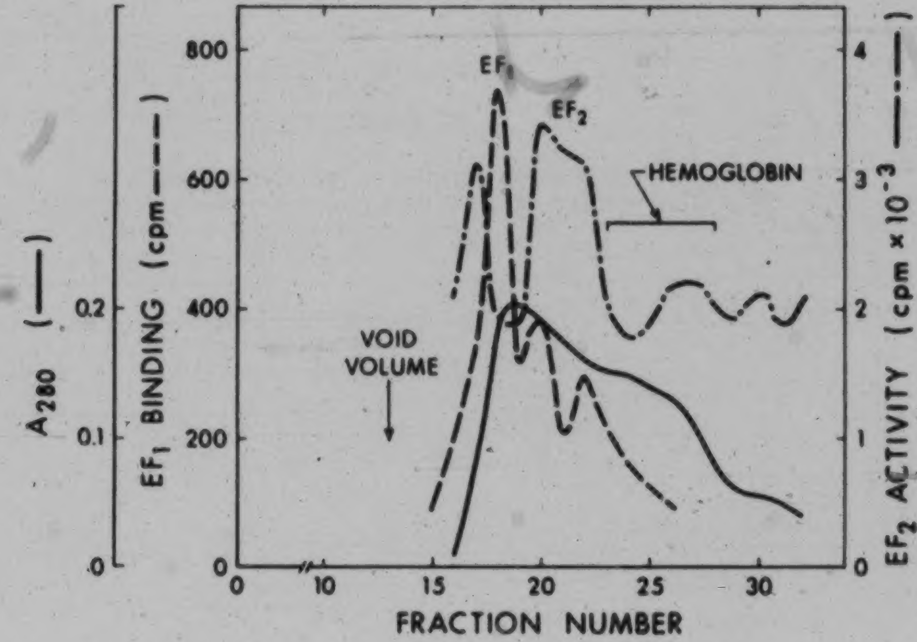


Figure 21. Isolation of peptide chain elongation factors by Sephadex G-100 gel filtration. Crude elongation factors (3 ml of 64 mg/ml)were placed on a 1.5 x 80 cm Sephadex G-100 column, previously equilibrated with 20 mM Tris-HCl (pH 7.4), 1 mM Mg^{2+}, 100 mM KCl, 1 mM DTE, 0.1 mM EDTA, 15% glycerol and fractionated as described in Materials and Methods. EF_1 activity was measured by the binding of $[^3H]$Phe-tRNA to twice washed ribosomes at 23° for 5 min in the presence of 5 mM Mg^{2+}. The EF_2 activity of the column fractions was assayed by measuring the extent of poly(U)-directed Phe polymerization in the presence of added EF_1 factor. EF_1 activity was corrected for non-enzymatic binding $[^3H]$Phe-tRNA to ribosomes (50 cpm) and EF_2 activity corrected for background counts at 0 time (200 cpm).

of EF_1 increased the binding of $[^3H]$Phe-tRNA to poly(U) programmed ribosomes 12-fold over the amount nonenzymatically bound. Two smaller peaks of aminoacyl-tRNA binding activity were observed after the major peak. Evidence for multiple forms of EF_1 has been observed with other mammalian sources (Schneir, Moldave, 1968; Collins *et al.*, 1972).

The activity of EF_2 was monitored by measuring the EF_1-dependent polymerization of $[^3H]$Phe in the cell-free system. The bulk of the activity was eluted in fractions 21-24 just prior to the hemoglobin peak (Fig. 21). Several other smaller peaks that stimulated Phe polymerization in the presence of EF_1 were observed, although the exact nature of these is unknown. Collins *et al.* (1972) also observed the elution of rat liver EF_2 before the hemoglobin peak on a Sepharose 6B column. The molecular weight of rat liver EF_2 has been reported to be 96,500 daltons ±9000 (Raeburn *et al.*, 1971).

In the following experiments, the EF_1 and EF_2 fractions were selected on the basis of minimum cross-contamination.

(ii) Requirements for Phe incorporation at 7.5 mM Mg^{2+} using *E. coli* $[^3H]$Phe-tRNA as substrate

Incorporation of Phe at various concentrations of EF_1 or EF_2 in the presence of saturating amount of the other was studied (Fig. 22). The results show that the amount of elongation factors required to saturate the system was approximately 40 μg protein of each fraction, and in their presence 80-90 pmoles [^{3}H]Phe were incorporated per mg enzyme per A_{260} unit of run-off 80S ribosome. Table 5 further defines the requirements for Phe incorporation at 7.5 mM Mg^{2+}. Ribosomes, EF_1, EF_2, GTP and poly(U) were essential. The addition of creatine phosphate plus creatine phosphokinase increased the level of incorporation by 30%. Possible mechanisms for this increase will be discussed later.

(iii) Effect of ribosome wash on Phe incorporation at 3.5 mM Mg^{2+}

The crude 0.5 M KCl ribosome wash stimulated Phe incorporation approximately 9-fold at low Mg^{2+} concentration (Table 6). Requirements for this reaction at 3.5 mM Mg^{2+} are outlined in Table 7. This incorporation was highly dependent on the presence of ribosomes, poly(U), and an energy source. As was observed earlier, the energy-generating system also contributed to the level of incorporation. Creatine phosphate and creatine phosphokinase are used to regenerate ATP from ADP which is required for acylation of tRNA. Since the present system relies on precharged tRNA, creatine phosphate and creatine phosphokinase should have little effect on [^{3}H]Phe incorporation. However, since the ribosome wash contains aminoacyl-tRNA synthetase activity the energy-generating system may be

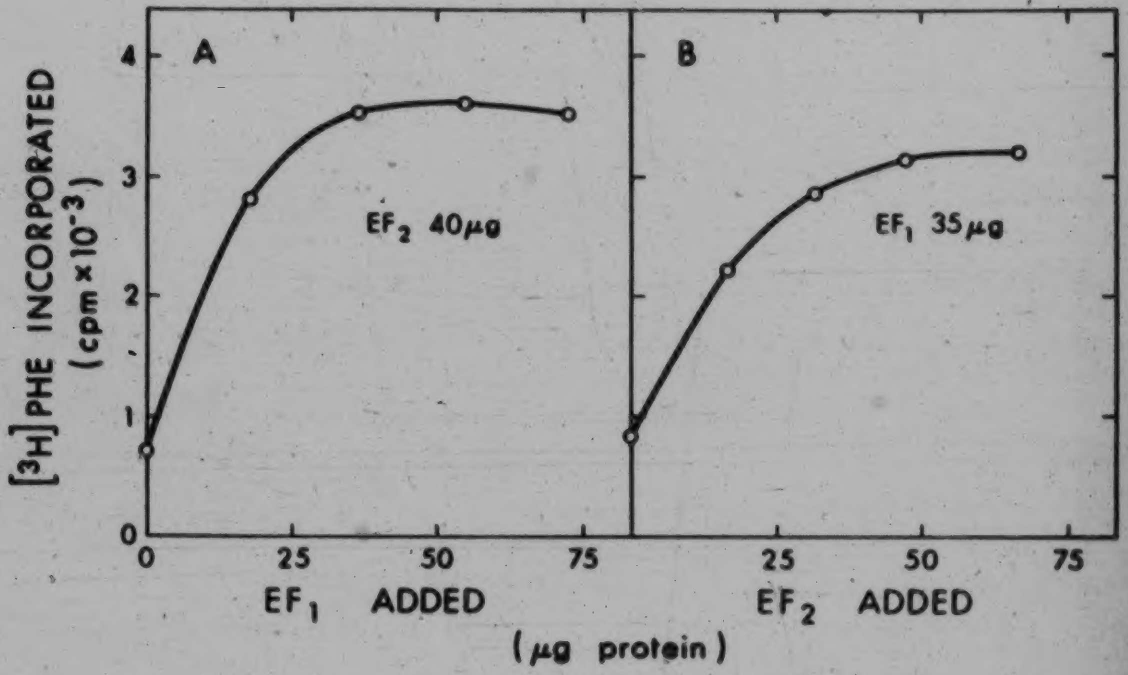


Figure 22. $[^3H]$Phe polymerization as a function of EF$_1$ and EF$_2$ concentration. The incorporation was carried out at 37° for 10 min in a volume of 100 μl which contained 6000 cpm *E. coli* $[^3H]$Phe-tRNA, 7.5 mM Mg^{2+} and other components as described in Materials and Methods. Background counts were not subtracted.

Table 5. Requirements for [^{3}H]Phe incorporation at 7.5 mM Mg^{2+} using *E. coli* charged tRNA as substrate

	[^{3}H]Phe incorporated/A_{260} ribosome	
Reaction mixture	pmoles	% control
Complete	8.9	100.0
-ATP, GTP	0.6	6.7
-GTP	0.9	10.3
-Creatine phosphate creatine phosphokinase	6.1	67.9
-poly(U)	1.2	14.0
-ribosomes	0.1	0.9
-EF_1	0.4	4.7
-EF_2	0.7	5.4
-EF_1 and EF_2	0.1	1.2

Incorporation, in a total volume of 100 µl was carried out at 37° for 10 min, in the presence of 35 µg EF_1, 40 µg EF_2, 3.5 pmoles *E. coli* [^{3}H]Phe-tRNA (equivalent to 17.5 pmoles/A_{260} ribosomes), and other components as described in Materials and Methods. Background at 0 time (0.25 pmoles) was subtracted.

Table 6. Stimulation of Phe incorporation by various amounts of 0.5 M KCl ribosome wash.

Ribosome wash (μg protein)	[^{3}H]Phe incorporated/A_{260} ribosome	
	pmoles	fold-stimulation
0	1.9	1.0
18	5.5	2.9
36	11.4	6.0
54	15.0	7.9
90	16.1	8.5
108	16.9	8.9
126	16.5	8.7

Incorporation in a total volume of 100 μl was carried out at 37° for 10 min, in the presence of 35 μg EF_1, 40 μg EF_2, 3.5 pmoles *E. coli* [^{3}H]Phe-tRNA (equivalent to 17.5 pmoles/A_{260} ribosome), 3.5 mM Mg^{2+} and other components as described in Materials and Methods. Background at 0 time (0.25 pmoles) was subtracted.

Table 7. Requirements for the stimulation of [^{3}H]Phe incorporation by two concentrations of 0.5 M KCl ribosome wash.

Reaction mixture	[^{3}H]Phe incorporated (pmoles)/A_{260} ribosome -ribosome wash	 +ribosome wash (30 µg)	 +ribosome wash (90 µg)
complete	1.9	5.6	12.5
-ATP, GTP	0.4	0.3	0.5
-GTP	0.6	1.6	11.3
-creatine phosphate creatine phosphokinase	2.0	3.8	9.6
-poly(U)	0.5	0.1	0.6
-ribosomes	0.0	0.0	0.0
-EF_1	0.9	4.1	13.7
-EF_2	0.7	3.0	13.2
-EF_1 and EF_2	0.0	3.6	12.2

Incorporation was carried out as described for Table 6.

utilized to recharge deacylated tRNA thereby increasing polymerization of Phe. Examination of the cold TCA-insoluble radioactivity (i.e. at 0^o which includes counts in both ^{3}H-polypeptides and [^{3}H]Phe-tRNA) after incubation revealed no increase in total counts. Therefore, there was no charging of tRNA with free [^{3}H]Phe ruling out the above possibility.

In regards to the requirements for energy no incorporation occurred in the absence of ATP and GTP (Table 7). However, in the absence of GTP variable amounts did occur. Dependence on GTP was much more pronounced at 30 μg of ribosome wash than at 90 μg, suggesting that the ribosome wash contained GTP.

The dependence of incorporation on added EF_1 and EF_2 was also abolished in the presence of 90 μg ribosome wash suggesting that the ribosome wash contained these factors. Elongation factors were shown to be present in ribosome wash from reticulocytes and Krebs ascites II cells (Shafritz *et al.*, 1970; Gilbert and Anderson, 1970; Metafora *et al.*, 1972).

The relationship between the amount of ribosome wash and the requirement for elongation factors was further investigated by two experiments. In the first, varying amounts of ribosome wash was added to two sets of reaction mixtures one of which contained added EF_1 and EF_2, the other did not (Fig. 23). At a wash concentration of 47 μg protein, Phe incorporation was no longer

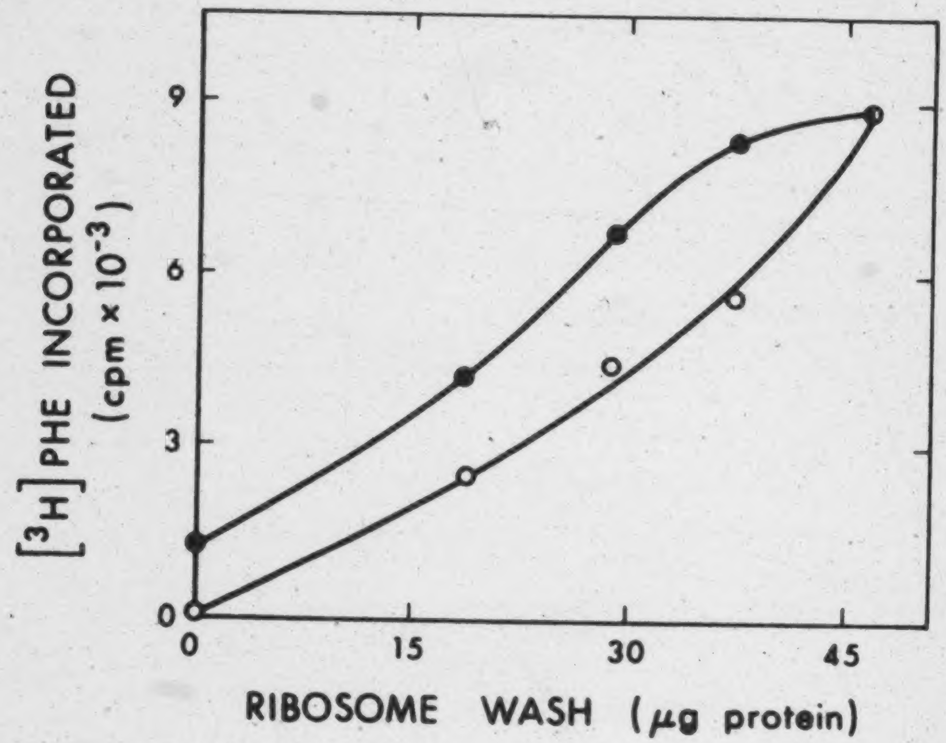


Figure 23. Incorporation of $[^3H]$Phe as a function of ribosome wash concentration in the presence and absence of EF_1 and EF_2. The incorporation was carried out at 37° for 10 min in a volume of 100 μl which contained 11,200 cpm *E. coli* $[^3H]$Phe-tRNA (2.8 pmoles), 3.5 mM Mg^{2+} and in the appropriate samples, 35 μg EF_1 and 40 μg EF_2. Background counts of 200 cpm were subtracted.

●——●, with EF_1 and EF_2

○——○, without EF_1 and EF_2

dependent on added EF_1 and EF_2. This clearly shows that saturating levels of ribosome wash (i.e. 65 μg, Fig. 18 and Table 6 contains sufficient elongation factors to support incorporation.

The second experiment involved selecting a fixed level of ribosome wash (0 μg or 30 μg), with varying amounts of elongation factors and observing the amount of Phe incorporated (Table 8). In the absence of ribosome wash with saturating amounts of elongation factors, the level of incorporation 1/3 that observed when only 30 μg ribosome wash was present (Table 8) and 1/8 when 45 μg was present (Fig. 23). This supports that conclusion that the ribosome wash contains an activity different from the elongation factors.

EF_1 and EF_2 were able to support 8.9 pmoles of $[^3H]$Phe incorporation at 7.5 mM Mg^{2+} (Table 5), but at 3.5 mM Mg^{2+}, ribosome wash was necessary to give an equivalent amount of incorporation (Table 8, line 5-7).

(iv) Phe incorporation by various liver ribosome preparations and different Phe-tRNAs

Using *E. coli* Phe-tRNA as substrate four different preparations of mouse liver ribosomes were tested for their ability to support $[^3H]$Phe incorporation at 3.5 mM Mg^{2+} (Table 9). Ribosome wash stimulated incorporation by all ribosomes, though not to the same extent. Run-off 80S and twice-washed ribosomes were similar in their response.

Table 8. [^{3}H]Phe incorporation as a function of added elongation factors and ribosome wash.

	Elongation Factors (µg protein)		[^{3}H]Phe incorporated/ A_{260} ribosome	
Ribosome wash (µg protein)	EF_1	EF_2	pmoles	fold stimulation
0	18	32	2.0	-
0	36	32	2.2	-
0	54	48	2.4	1.0
30	0	0	6.5	2.7
30	18	32	8.8	3.6
30	36	32	8.9	3.7
30	54	48	9.0	3.7

Incorporation was carried out at 37° for 10 min in the presence of 3.5 mM Mg^{2+}, 2.8 pmoles *E. coli* [^{3}H]Phe-tRNA (equivalent to 14.0 pmoles/A_{260} ribosome), and other components as described in Materials and Methods. Background at 0 time (0.25 pmole) was subtracted.

Table 9. [^{3}H]Phe incorporation by various preparations of mouse liver ribosomes.

	[^{3}H]Phe incorporated (pmoles)/A_{260} ribosome		
Ribosomes	-ribosome wash	+ribosome wash	fold stimulation
Run-off ribosomes	2.0	6.4	3.1
Twice-washed membrane-bound ribosomes	4.5	8.3	1.9
Twice-washed ribosomes	1.7	5.6	3.3
40S + 60S subunits	2.7	5.1	1.9

Incorporation was carried out at 37° for 10 min, in the presence of 35 μg EF_1, 40 μg EF_2, 30 μg ribosome wash, 0.2 A_{260} ribosomes, and 2.8 pmoles *E. coli* [^{3}H]Phe-tRNA and other components as described in Materials and Methods. Ribosome subunits were present in a 40S/60S A_{260} ratio of 1/2.5. Background at 0 time (0.25 pmole) was subtracted.

Surprisingly the 40S + 60S ribosomes had a higher background activity (in the absence of ribosome wash) than run-off 80S and consequently did not respond as well.

Substrate specificity was examined in an experiment in which tRNAs from different sources were tested. *E. coli*, yeast and mouse liver tRNA were charged with [^{3}H]Phe as described in the Materials and Methods. In the absence of ribosome wash, liver and yeast tRNA showed an optimum Mg^{2+} concentration for polypeptide synthesis of 8.5 mM while *E. coli* tRNA was at 6.5 mM Mg^{2+} (Fig. 24).

Addition of liver ribosome wash resulted in decreases in the optimum Mg^{2+} concentration to 6.5 mM Mg^{2+} for liver and yeast tRNA and to 5.5 mM Mg^{2+} for *E. coli* tRNA. With all three types of tRNA the maximum stimulation by ribosome wash was obtained at 3.5 mM Mg^{2+}.

(c) DEAE-cellulose chromatography of mouse liver ribosome wash

Crude 0.5 M KCl ribosome wash was fractionated on a DEAE-cellulose column (1.0 x 25 cm) prepared as described in Materials and Methods. Routinely, 100-150 mg protein was placed on the column and eluted with 10 mM Tris-HCl (pH 7.4) buffer containing 1 mM Mg^{2+}, 50 mM KCl, 0.1 mM EDTA, and 1 mM DTE. When the effluent A_{280} approached the background level, a linear gradient of 0.05 M to 0.4 M KCl in the above buffer was started. As shown in Figure 25, the column effluent was divided into nine fractions and

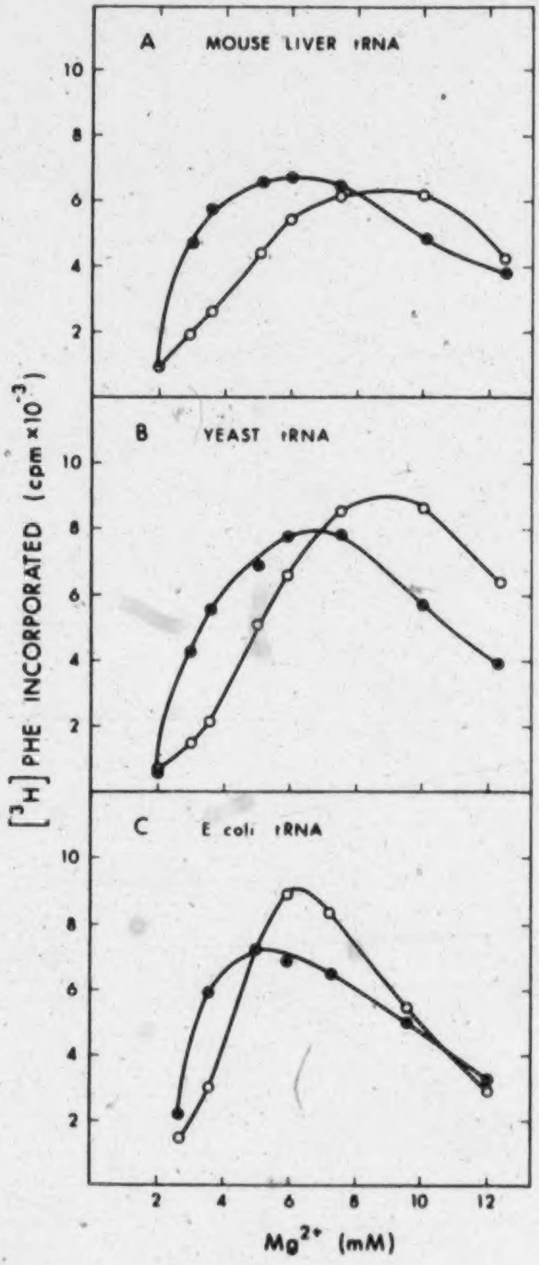


Figure 24. Polymerization of [^{3}H]Phe at various Mg^{2+} concentrations with and without ribosome wash using charged tRNA from *E coli*, yeast and mouse liver. The incorporation was performed at 37° for 10 min in the presence of 35 µg EF$_1$, 40 µg EF$_2$, 90 µg liver ribosome wash and other components as described in the Materials and Methods. Aminoacylated tRNA was present in the following amounts; 16,900 cpm mouse liver [^{3}H]Phe-tRNA, 16,000 cpm yeast [^{3}H]Phe-tRNA and 14,000 cpm *E. coli* [^{3}H]Phe-tRNA. ●—●, with ribosome wash; ○—○, without ribosome wash.

and assayed singly or in combination for the ability to support Phe incorporation at 3.5 mM Mg^{2+}. The results of a typical experiment are shown in Table 10. The three most active fractions were: fraction I eluted with 0.05 M KCl; fraction VI eluted between 0.18-0.25 M KCl; and fraction VIII eluted between 0.31-0.38 M KCl. Highest stimulation of incorporation was usually obtained when all three fractions were present, although in a few experiments fraction I plus VIII, and VI plus VIII also gave high values. The maximum stimulation was about 50% of that obtained with crude wash.

The 0.05 M KCl pass-through fraction appeared to be similar to IF-M_1 obtained from rabbit reticulocyte (Shafritz, Anderson, 1970; Woodley *et al.*, 1972) and rabbit liver (Picciano *et al.*, 1972) ribosome wash. The two remaining fractions, VI and VIII were initially thought to correspond to the reticulocyte factors IF-M_3 (eluted at 0.17 M KCl) and IF-M_2 (eluted at 0.26-0.32 M KCl) respectively, as described by Anderson and his coworkers. But in the reticulocyte system only IF-M_1 and IF-M_2 were required for poly(U) translation at low Mg^{2+} concentration. In our system, highest stimulation frequently occurred when all three fractions were added.

More recent results using fractionated rabbit liver ribosome wash, revealed IF-M_2 to have a much broader peak (0.2-0.4 M KCl, Picciano *et al.*, 1972) than the corresponding reticulocyte factor (0.26-0.32 M KCl). A

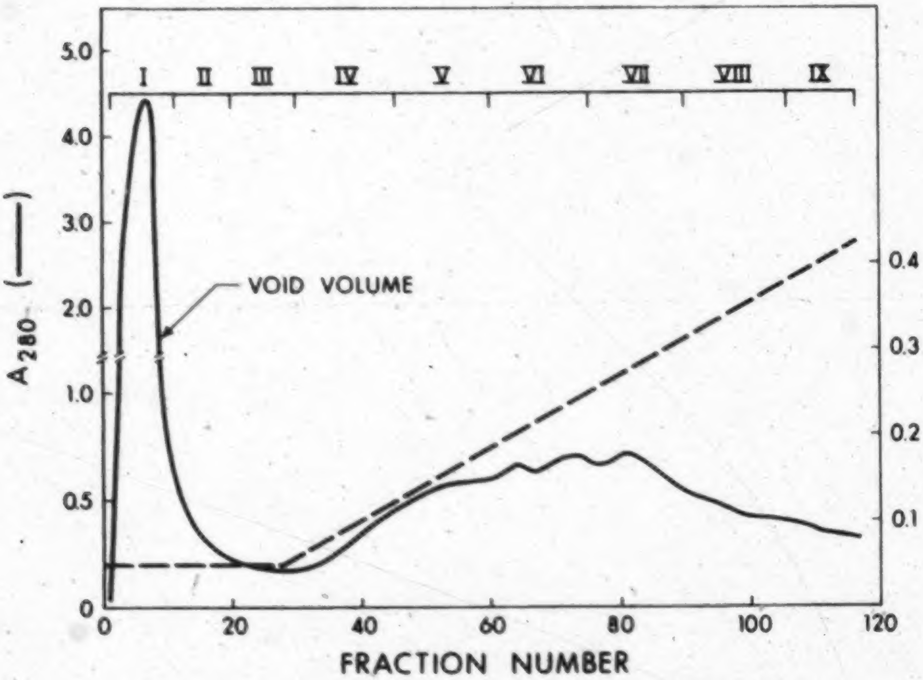


Figure 25. DEAE-cellulose chromatography of 0.5 M KCl ribosome wash. Ribosome wash (10-15 ml, 9 mg protein/ml) was placed on a 1.5 x 25 cm DEAE-cellulose column previously equilibrated with 10 mM Tris-HCl (pH 7.4), 1 mM Mg^{2+}, 50 mM KCl, 0.1 mM EDTA, 1 mM DTE and fractionated as described in Materials and Methods. Absorbance at 280 nm was determined using a portion of each fraction. Linearity of the KCl gradient was verified by measuring conductivity.

Table 10. Stimulation of [^{3}H]Phe incorporation by partially purified ribosome wash factors.

	[^{3}H]Phe incorporated/A_{260} ribosome		
Fractions	pmoles	-background	% control
- Crude ribosome wash	2.0	-	-
+ Crude ribosome wash	8.9	6.9	100.0
I (30 μg)	2.0	0.0	0.0
VI (15 μg)	2.2	0.2	2.9
VIII (15 μg)	2.6	0.6	8.7
I + VI	2.8	0.8	11.6
I + VIII	3.7	1.7	24.7
VI + VIII	3.9	1.9	27.6
I + VI + VIII	5.2	3.2	46.4

Incorporation, in a volume of 100 μl was carried out at 37° for 10 min in the presence of 35 μl EF_1, 40 μg EF_2, 90 μg crude ribosome wash, 0.1 A_{260} run-off 80S ribosomes, 2.5 pmoles *E. coli* [^{3}H]Phe-tRNA, 4 mM Mg^{2+} and other components as described in Materials and Methods. Background at 0 time (0.37 pmole) was subtracted.

possible explanation of our data is that fraction VI in fact contains a considerable amount of IF-M_2 factor. The reason for the high stimulatory activity of VI and VIII combined is not clear at present.

D. Discussion

It was shown in the preceding Chapter that the ribosome subunits of mouse liver as prepared were physically intact. In the present study they were found to incorporate Phe as actively as run-off 80S ribosomes and were most active at a 40S/60S A_{260} ratio of 1/2.5, incorporating 10-15 Phe residues per 80S ribosome. These subunits also showed little endogenous activity.

Stability of the mRNA-peptidyl-ribosome complex seems to vary depending on the type of tissue from which it was isolated. For example, polysomes from mouse plasmacytoma tumor cells dissociated into subunits readily at 1 mM Mg^{2+} and 100 mM KCl (Mechler, Mach, 1971). The subunits thus obtained were highly active, supporting the incorporation of 10-25 Phe residues per ribosome. To obtain this activity however, an A_{260} ratio of 40S/60S of 1/1 was shown to be required which suggests that 60% of the 40S subunits were inactive. Others have also observed the smaller mammalian ribosome subunit to be less stable than the larger (Tashiro, Siekevitz, 1965; Hamilton, Ruth, 1969).

The activity of mammalian ribosome subunits prepared by several groups is shown in Table 11. The number of phenylalanine residues incorporated per ribosome is also indicated, although some caution must be exercised in directly comparing these figures. Differences in reaction conditions, such as temperature, length of incubation and whether the concentration of various components was optimized, will result in variations of incorporation. With this in mind these numbers can be used as a general indicator of the activity of the ribosome subunits produced by various methods. In most of the preparations the 60S subunit fraction alone was capable of considerable Phe incorporation due to contamination with 40S or 60S particles.

The mouse liver cell-free incorporating system using run-off 80S ribosomes and precharged tRNA was found to be well-suited for the study of protein synthesis. The system was dependent on added poly(U), ribosomes, GTP, elongation factors (Table 5) and had a low background activity. Both liver and *E. coli* [^{14}C]Phe-tRNA served as substrate in poly(U)-directed Phe incorporation. Shafritz and Anderson (1970) have observed Phe-tRNA from *E. coli* and rabbit reticulocytes to function as substrate for polyphenylalanine synthesis in a rabbit reticulocyte cell-free system. Efficiency of utilization of our tRNA substrate was equivalent to that reported for rat liver (Moldave, 1968), mouse brain (Gilbert, Johnson, 1972) and rabbit reticulocyte (Shafritz *et al.*, 1970) cell-free systems.

Table 11. Poly(U)-directed [^{14}C]Phe incorporation by various mammalian ribosome subunits

Source of ribosomes	[^{14}C]Phe incorporated (% of 80S control)			Optimum 40S/60S A_{260} ratio	Phe residues incorporated per ribosome
	40S	60S	40S + 60S		
Rat muscle[a]	3.6	24.8	110	1/2*	2-7
Rat liver[b]	9.0	22.0	105	1/2*	<1
Rat liver[c]	2.0	9-24	50-80	1/2*	1-2
Rat & mouse liver[d]	2.6	15.0	70	1/2	15-20
Mouse liver[e]	1.0	2.0	108	1/2.5	10-15
Mouse plasma-cytoma tumor[f]	1.0	†	100	1/1	15-25
Rabbit reticulocytes[g]	1.0	4.0	100	1/2.5*	3-6

Ribosome subunits prepared by: a) Martin *et al.*, (1969), b) Lawford, (1969), c) Petermann, Pavlovec, (1971), d) Falvey, Staehelin, (1970), e) Faber, (this thesis), f) Mechler, Mach, (1971), g) Busiello *et al.*, (1971). *Optimum 40S/60S A_{260} ratio was not determined; subunits were mixed for incorporation studies at the ratio as indicated. † Value was not given; Mechler and Mach just indicated that the 60S subunits had considerable incorporating activity.

Sephadex G-100 fractionation of the crude elongation factors produced two main peaks of activity. EF_1 eluted after the void volume and EF_2 before the hemoglobin band. Both fractions were required for Phe incorporation into hot TCA-insoluble polypeptides. After the main EF_1 peak, one or two smaller peaks of aminoacyl-tRNA binding activity were observed which varied in size and distribution from one preparation to another. A possible explanation for this could be that these are aggregates of EF_1 as has been reported by Collins *et al.* (1972) for rat liver EF_1. Alternatively, the minor peaks of binding activity could represent IF-M_1 present in the crude elongation factor preparation. Shafritz and Anderson (1970) have shown that rabbit reticulocyte IF-M_1 promoted the binding of both N-acetyl-Phe-tRNA and Phe-tRNA to washed reticulocyte ribosomes. They also observed EF_1 to elute before IF-M_1 on a Sephadex G-150 column. Picciano *et al.* (1973) determined the molecular weight of reticulocyte IF-M_1 to be 96,000 daltons, which would place it on our Sephadex G-100 profile (Fig. 21) approximately between the hemoglobin band and the major EF_1 peak.

Further caution must be exercised in interpreting [^{14}C]Phe-tRNA binding data. McKeehan *et al.* (1970) observed a factor in reticulocyte 0.5 M KCl ribosome wash which could bind N-Ac-Phe-tRNA to itself or to washed ribosomes. This binding was independent of poly(U) and GTP, with the complex being retained on a nitrocellulose filter. The binding activity remained coincident with the Phe activating enzyme throughout several purification steps. These results suggest that Phe-tRNA synthetase present in the ribosome wash promoted the binding of [^{14}C]Phe-tRNA. The crude elongation factor preparation that we used contained some aminoacyl-tRNA synthetase activity, however, assay of our Sephadex G-100 fractions showed that activity to acylate liver tRNA with [^{14}C]Phe was in the void volume and not coincident with our EF_1 peak. This provides further evidence that the factor separated by Sephadex G-100 filtration was, in fact, the aminoacyl-tRNA binding protein.

The reason for the increase in incorporation activity by the addition of the ATP-generating system (creatine phosphate, creatine phosphokinase) is not clear. Shafritz and Anderson (1970) also reported a 30% increase in polyphenylalanine synthesis by the addition of pyruvate kinase and phosphoenolpyruvate (PK + PEP) to a reticulocyte cell-free system. Similarly Collins, Moon, and Maxwell (1972) reported 2- to 3-fold stimulation of Phe incorporation by PK + PEP at 10 mM Mg^{2+}. They suggest that PK + PEP catalyzes phosphorylation of GDP (James, Morrison, 1966) and thus prevents the buildup of GDP which might inhibit protein synthesis by formation of an EF_2-GDP-ribosome complex (Skogerson, Moldave, 1968).

Our results confirm those of others (Miller, Schweet, 1968; Shafritz *et al.*, 1970; Grummt, 1970; Means *et al.*, 1971; Picciano *et al.*, 1973) that ribosomal wash contains protein factors capable of initiating polyphenylalanine synthesis at low Mg^{2+} concentration. The amount of crude ribosome wash to saturate the charged tRNA system (65 μg) was approximately one-half that required by the Nirenberg system (150 μg). Thus the lower protein content of the charged tRNA system appears to increase the sensitivity, but the reason is not clear at present.

The broad specificity of the initiation factors IF-M_1 and IF-M_2 was illustrated when liver ribosome wash produced a "Mg^{2+} shift" in the mouse liver system using *E. coli* Phe-tRNA as substrate. In the original *E. coli* poly(U)-dependent system, both N-acetyl-Phe-tRNA and Phe-tRNA were required in the presence of IF-1 and IF-2 to shift the Mg^{2+} optimum (Lucas-Lenard, Lipmann, 1966). Lack of requirement for an N-blocked amino acid in our system can be explained by the fact that in eucaryotes the initiator tRNA, Met-$tRNA_F$ is not formylated (Smith, Marcker, 1970). For the purpose of assay of these factors it is convenient that *E. coli* Phe-tRNA can function in the liver cell-free system, but the biological significance remains to be determined.

The results so far presented suggest that the ribosome wash contains factors which initiate the translation of poly(U). In the following Chapter evidence will be presented that the ribosome wash is required for translation of exogenous liver mRNA.

CHAPTER IV

ISOLATION OF MOUSE LIVER mRNA AND ITS TRANSLATION IN A HOMOLOGOUS CELL-FREE SYSTEM

A. Introduction

Liver tissue contains two types of polysomes in the cytoplasm which are involved in protein syntheis. Early attempts *in vivo* and subsequent work using isolated polysomes have provided evidence that free polysomes synthesize proteins for intracellular use (e.g. ferritin) while those attached to the endoplasmic reticulum synthesize extracellular proteins ((e.g. serum albumin), Siekevitz, Palade, 1960; Birbeck, Mercer, 1961; Peters, 1962; Takagi, Ogata, 1968; Ganoza, Williams, 1969; Redman, 1969). Extracellular proteins are believed to migrate from the rough endoplasmic reticulum to the smooth endoplasmic reticulum (Peters, 1962), possibly to the Golgi apparatus and eventually are excreted from the cell by a process of reverse pinocytosis. The synthesis of albumin amounts to about one-third of the total protein produced by liver hence considerable interest has been focused on the structure and function of albumin. Recently, this topic was reviewed by Peters (1970).

An initial attempt for the cell-free synthesis of albumin under the direction of exogenous mRNA employed rat liver RNA extracted from albumin-synthesizing polysomes which had been precipitated by anti-albumin serum (Uenoyama, Ono, 1972). It was found however that the albumin mRNA fraction thus obtained was partially degraded, and was only capable of producing short polypeptide chains although they were still reactive with anti-albumin antibody. Evidence for the cell-free production of the complete albumin molecule has so far been lacking.

Most mRNAs from mammalian cells contain a segment of polyadenylic acid (Kates, 1970; Lee *et al.*, 1971; Darnell *et al.*, 1971; Edmonds *et al.*, 1971; Mendecki *et al.*, 1972) with the possible exception of histone mRNA (Adesnik, Darnell, 1972). These segments contain 150-200 adenine residues which allow their selective binding to Millipore filters (Rosenfeld *et al.*, 1972), as well as poly(U) (Sheldon *et al.*, 1972) and oligo d(T) (Armstrong, *et al.*, 1972) covalently linked to cellulose. Recently, cellulose was found capable of binding synthetic poly(A) at high ionic strength (Kitos *et al.*, 1972). Schutz *et al.* (1972) made use of this observation for the isolation of intact globin and ovalbumin mRNA.

The function of poly(A) in mRNA is not clear but since it is added post-transcriptionally (Darnell *et al.*, 1971), it may be involved in the processing of mRNA. An exception was the finding by Adesnik and Darnell (1972) that histone mRNA did not contain a poly(A) segment. Lack of poly(A) may confer special properties on the mRNA and it has been noted that histone mRNA appears in polysomes earlier than poly(A)-containing mRNA (Schochetman, Perry, 1972) and seems to have a shorter lifetime than other mRNAs (Borun *et al.*, 1967; Craig, *et al.*, 1971). This suggests that poly(A) may be involved in controlling transport of mRNA from the nucleus to the cytoplasm or possibly alters the mRNA lifetime by changing its stability.

Experiments described in this Chapter provide evidence for the synthesis of albumin directed by exogenous mouse liver mRNA in the homologous cell-free system. Messenger RNA extracted from total and membrane-bound polysomes by two different methods directed the synthesis of albumin. Further fractionation of RNA by cellulose chromatography and sucrose density-gradient centrifugation was examined. A cell-free amino acid incorporation system utilizing run-off 80S ribosomes was developed which was strictly dependent on mRNA and protein initiation factors. Analysis of the products by polyacrylamide gel electrophoresis and immunoprecipitation showed that newly-formed albumin could account for up to 8% of the total protein synthesized.

B. Materials and Methods

Sigmacell type 38 microcrystalline cellulose, cycloheximide and sodium dodecyl sulfate were purchased from Sigma Chemical Co.; phenol and Amido Black from J. T. Baker Chemical Co.; aurintricarboxylic acid, disodium ethylenediaminetetraacetate and ammonium persulfate from Fisher Scientific Co.; sodium deoxycholate and vitamin B_2 from Matheson, Coleman and Bell; acrylamide, N, N'-methylenebisacrylamide and N, N, N', N'-tetramethylethylenediamine from Eastman Kodak Co.; Aquasol scintillation fluid from New England Nuclear; mouse serum, chicken serum, rabbit antiserum to chicken serum, and rabbit antiserum to mouse albumin from Nutritional Biochemicals Corporation; [^{14}C]Leu, 342 mCi/mmole and ^{14}C-protein hydrolysate 57 mCi/milli atom carbon from Amersham/Searle.

All buffers and glassware used for the preparation of RNA were autoclaved before use. Plastic gloves were used in all critical handling procedures to prevent possible RNase contamination.

Ribosomes

Run-off 80S ribosomes required for amino acid incorporation were prepared as described in Chapter II. Some modifications were introduced in the preparation of membrane-bound and total polysomes. They included: doubling the volume of homogenizing buffer from 2.5 ml to 5.0 ml buffer/g liver, slower homogenization speed (approximately 400 rpm), and gentle handling of the post-mitochondrial supernatant by large-bore pipettes. Large polysomes were prepared in this way without bentonite.

Extraction of polysomal RNA

RNA was extracted with phenol by the method of Schutz *et al.*, (1972) with minor modifications. Total polysomes or membrane-bound polysomes were treated with 1%

DOC in the presence of post-ribosomal supernatant. The polysome pellets were resuspended in 20 mM Tris-HCl (pH 8.3), 5 mM EDTA, 75 mM NaCl, 0.5% SDS and an equal volume of phenol (saturated with the same buffer) was added. The mixture was shaken for 10 min at room temperature and centrifuged at 10,000 x g (av) for 10 min. The aqueous layer was removed, and the phenol layer reextracted with buffer. The pooled aqueous phase was extracted twice more with phenol. The final aqueous layer was adjusted to 0.3 M LiCl; 2.5 volumes of 95% ethanol was added and the sample stored overnight at -30°. RNA precipitated was collected by centrifugation, dissolved in a minimal volume of 20 mM Tris-HCl (pH 7.4) containing 20% glycerol and reprecipitated. The final precipitate was washed three times with ethanol and dissolved in Tris-glycerol buffer at a concentration of 100-200 A_{260}/ml.

Polysomal RNA was also extracted by an SDS-EDTA method (Rhoads *et al.*, 1971). The polysome pellets were resuspended in SDS buffer (40 mM Tris-HCl (pH 8.3), 5 mM EDTA, 20 mM sodium acetate, 1.0% SDS) and incubated at 37° for 5 min. Seventy-five A_{260} units (4 ml) were layered on linear 10 to 30% sucrose gradients (30 ml in SDS buffer), and centrifuged in a Spinco SW 27 rotor at 22,000 rpm for 14 hrs at 23°. Recording of the optical density profile and recovery of the gradient fractions was as described in Chapter II. Fractions corresponding to 15-20S RNA were pooled, 2.5 volumes of 95% ethanol was added followed by NaCl to give a final concentration of 0.1 M. RNA precipitated was washed with ethanol as described above, dissolved in Tris-glycerol buffer and stored in small amounts in liquid N_2.

Cellulose chromatography of polysomal RNA

Polysomal RNA (100-600 A_{260} units) was diluted 10-fold with buffer containing 20 mM Tris-HCl (pH 7.4), 0.2 mM Mg^{2+}, 500 mM KCl and applied to a 1 x 6 cm cellulose column (1 g dry weight) previously equilibrated with the same buffer as described by Schutz *et al.*, (1972). The column was washed with high salt buffer until the wash gave a reading less than 0.01 A_{260}, then eluted with 4 mM Tris-HCl (pH 7.4) collecting 1 ml fractions. Absorbance at 260 nm was measured and peak fractions were pooled and RNA recovered by ethanol precipitation. In some cases the pooled fraction was dialyzed briefly against 20 mM Tris-HCl (pH 7.4) 20% glycerol and stored in small amounts in liquid N_2.

Cell-free translation of mRNA

Protein synthesis was measured in a cell-free amino acid incorporation system utilizing run-off 80S ribosomes. The reaction mixture for each assay contained in 100 µl: 0.1 µmole ATP, 0.03 µmole GTP, 1 µmole creatine phosphate, 5 µg creatine phosphokinase , 12.5 µg mouse liver tRNA, 0.2 A_{260} unit of mouse liver run-off 80S ribosomes, 600 μg supernatant protein, 150 μg ribosome wash protein, 0.25 μCi [^{14}C]Leu and 5 mμmoles of each of 19 cold amino acids, 20 mM Tris-HCl (pH 7.8), 3.5 mM $MgCl_2$, 65 mM KCl, 6 mM β-ME and 0.5 mM DTE. The amount of RNA was 10 μg per assay for cellulose-column purified RNA, and 50 μg for 15-20S RNA. The reaction was carried out at 30° for the times indicated in the figure legends. Hot TCA-insoluble radioactivity was determined as described in Chapter III.

Analysis of cell-free products by polyacrylamide gel electrophoresis.

When analysis of translational products was intended [^{14}C]Leu was substituted by ^{14}C-protein hydrolysate (7.5 μCi/ml reaction mixture) containing 15 labeled L-amino acids. Cold methionine, tryptophan, asparagine, glutamine and cysteine were supplemented at 50 mμmoles/ml. Reaction mixture components for incorporation were scaled up 10-fold and in some cases 50-fold. Samples were incubated at 30° for 60 min and centrifuged at 149,000 x g (av) for 90 min. The supernatant was removed and subjected to polyacrylamide gel electrophoresis as described under Materials and Methods in Chapter II. After electrophoresis was completed, the gels were stained with Amido Black (1% by weight in 7.5% acetic acid), destained electrophoretically, scanned at A_{650} to locate the protein bands, and then sliced into 2 mm fractions. Each fraction was placed in a counting vial and dissolved in 30% H_2O_2 by incubating at 60° overnight. Ten ml of Aquasol scintillation fluid was added under reduced light and the samples were cooled for 4 hours at 4° to reduce chemiluminescence. Under these conditions a background count of 40 cpm was observed.

Partial purification of albumin after cell-free translation of liver mRNA

Translation of mRNA was carried out in the cell-free system as described previously in a total volume of 5 ml at 30° for 60 min. The sample was centrifuged at 149,000 x g (av) for 90 min. The supernatant was adjusted to pH 4.8 and the precipitate removed by centrifugation at 12,000 x g (av) for 10 min. $(NH_4)_2SO_4$ was added to the supernatant to 40% saturation at 0°. The resulting precipitate was collected as above, washed twice with 40% $(NH_4)_2SO_4$ in 0.02% potassium acetate (pH 4.8), and dissolved in 50 mM Tris-HCl (pH 8.0), 6 mM β-ME and dialyzed against the same buffer for 2 hr. The sample was then heated at 60° for 5 min. Aggregated protein was removed by centrifugation and the supernatant was concentrated, if necessary, by B15 Minicon Macrosolute Concentrator (Amicon Corp., Lexington, Mass.) and analyzed by gel electrophoresis. Albumin was purified 3- to 4-fold by this procedure as assayed by gel electrophoresis.

C. Results

1. Isolation of mouse liver polysomal RNA

(a) Distribution of RNA between free and membrane-bound polysomes

It has been shown that vigorous homogenization conditions can increase the yield of endoplasmic reticulum by fractionating it into smaller fragments (Blobel, Potter, 1967). Since it is important, in this study, to isolate intact polysomes mild homogenization was used at a possible expense of polysome yield. Table 12 shows the distribution of polysomes obtained under our conditions. The yield of total polysomes or free and membrane-bound polysomes was 35-40 A_{260} units/g liver. Initially total polysomes were used as the source of RNA because of the ease of obtaining large polysomes in the range of 10-20 80S ribosomes per aggregate. In a later stage of the study membrane-bound polysomes prepared without detergent were also used.

(b) Sucrose density-gradient fractionation of polysomal RNA

Total polysomal RNA extracted with SDS-phenol was fractionated by sucrose density-gradient centrifugation. Figure 26 shows the sedimentation pattern of 50 A_{260} units of RNA analyzed on a 30 ml linear 10-30% sucrose gradient. Samples were taken from the various fractions and tested for their ability to direct [^{14}C]Leu incorporation in the cell-free system. The highest incorporating activity was

Table 12. Distribution of mouse liver RNA between free and membrane-bound polysomes.

Polysomes	A_{260}	A_{280}	A_{260}/A_{280}	A_{260} units/g liver
Free	0.29	0.17	1.70	15.3
Bound	0.42	0.23	1.75	20.5
Total	0.70	0.40	1.78	38.0

Free, membrane-bound, and total polysomes were prepared as described in the Methods. Membrane-bound polysomes were released from the membranes by DOC treatment and recovered by pelleting through 2 M sucrose buffer at 176,000 x g (av) for 5 hours.

found in the 18S RNA fraction. Since albumin mRNA has a presumptive sedimentation coefficient of 18S fractions sedimenting at 15-20S RNA were pooled and RNA was recovered by ethanol precipitation.

(c) Cellulose column chromatography of SDS-phenol extracted RNA

RNA was extracted from total polysomes with SDS-phenol and chromatographed on cellulose. Figure 27 shows the elution profile from the column. RNA retained by the column at high ionic strength and subsequently eluted with 4 mM Tris-HCl (pH 7.4) buffer represented about 2% of the applied material. Tris buffer was as effective as distilled water in the elution of RNA. Table 13 lists the results of several experiments in which 100 to 600 A_{260} units of RNA from total and membrane-bound polysomes were chromatographed. Approximately 2-3% of total polysomal RNA was retained on cellulose at high ionic strength as compared to 5-7% of the membrane-bound polysomal RNA.

Sucrose density-gradient analysis of the cellulose pass-through and cellulose-retained polysomal RNA is shown in Figure 28. Cellulose pass-through RNA produced a sedimentation profile typical of that of mammalian ribosomal RNA containing a 5S, 18S and 28S component. The cellulose-retained RNA, on the other hand, showed a major component sedimenting at 14-16S, and two minor peaks at 11S and 25S.

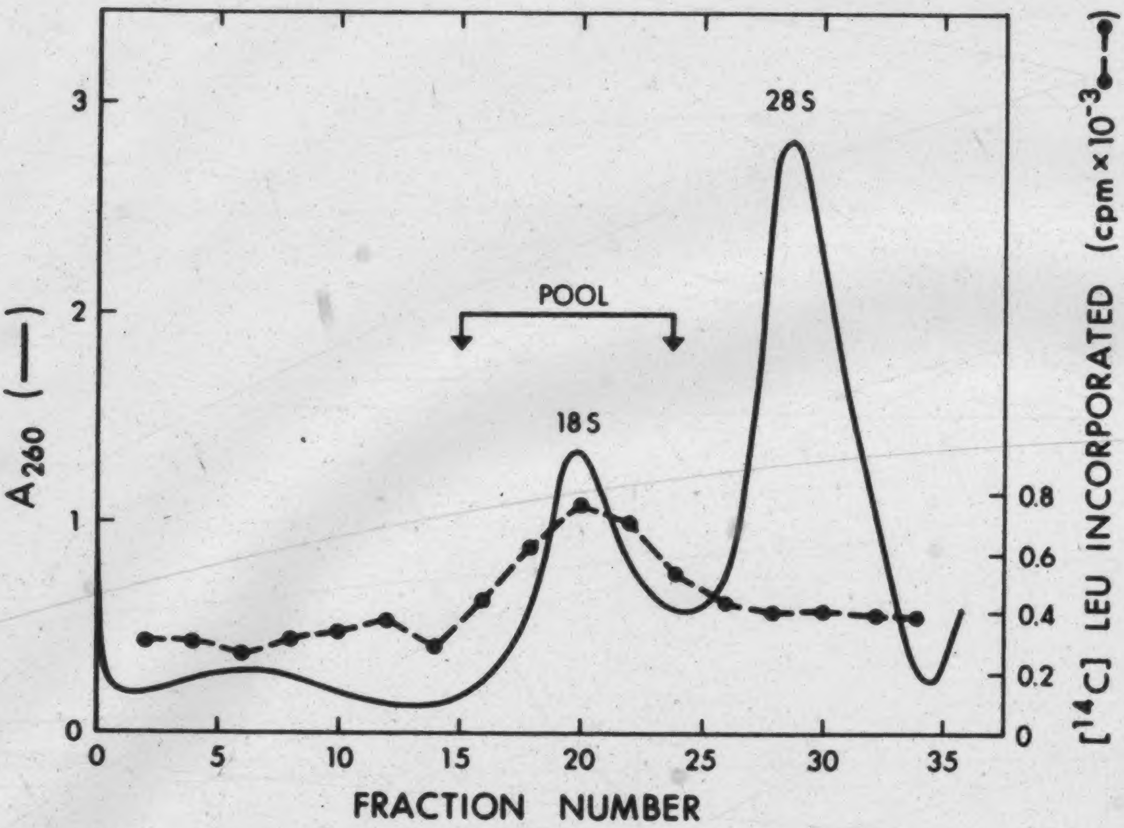


Figure 26. Fractionation of polysomal RNA by sucrose density-gradient centrifugation. Polysomal RNA (50 A_{260} units) extracted from total polysome by the SDS-phenol method was layered on a 30 ml 10-30% sucrose gradient containing 20 mM Tris-HCl (pH 7.4), 1 mM Mg^{2+} and 50 mM NaCl. Centrifugation was in a Spinco SW 27 rotor at 22,000 rpm for 14 hr at 4°. Fractions of approximately 0.9 ml were collected as described in Materials and Methods, Chapter II. Samples (10 µl) from the various fractions were tested for their ability to direct [^{14}C]Leu incorporation in the cell-free system.

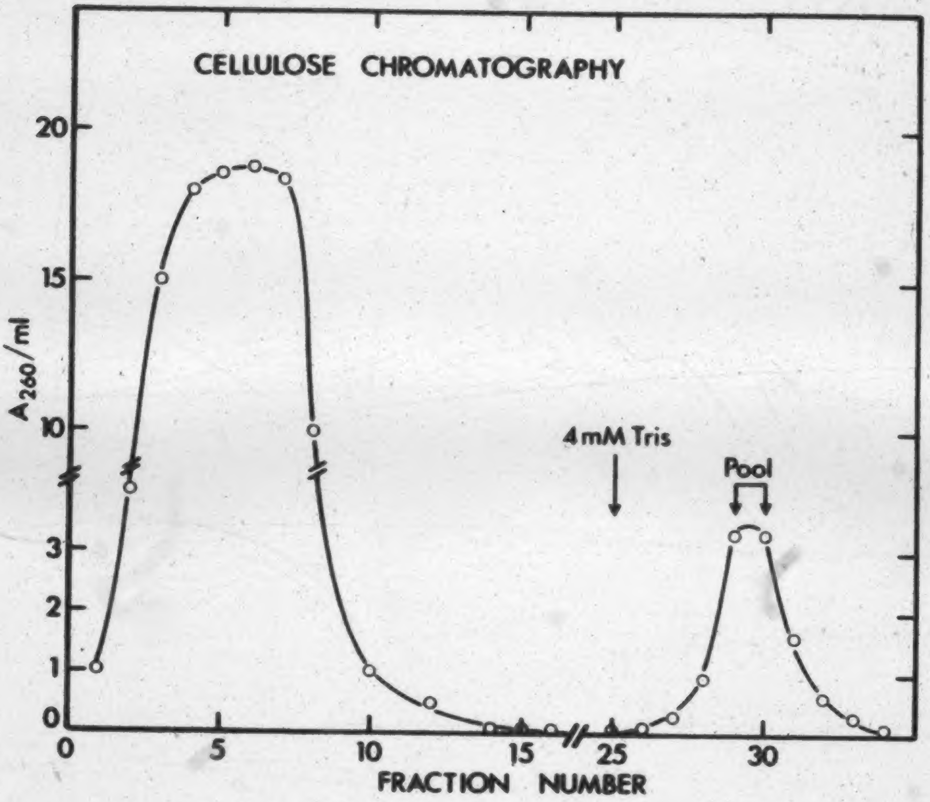


Figure 27. Cellulose column chromatography of polysomal RNA. Polysomal RNA (225 A_{260} units) extracted from total polysomes was diluted 10-fold with buffer containing 20 mM Tris-HCl (pH 7.4), 0.2 mM Mg^{2+}, 500 mM KCl and applied to a 1 x 6 cm cellulose column previously equilibrated with the same buffer. The column was extensively washed with high salt buffer, then poly(A)-containing RNA eluted with 4 mM Tris-HCl (pH 7.4) buffer as described in Materials and Methods.

Table 13. Binding of poly(A)-containing polysomal RNA to cellulose at 0.5 M KCl.

RNA sample	A_{260} units applied	A_{260} units eluted with 4 mM Tris-HCl	% RNA bound
Total polysomal	115	2.3	2.0
Total polysomal	225	6.6	2.9
Total polysomal	316	11.0	3.4
Total polysomal	375	6.5	1.7
Total polysomal	600	18.5	3.0
Total polysomal	653	15.0	2.3
Membrane-bound polysomal	220	11.4	5.2
Membrane-bound polysomal	250	18.0	7.2

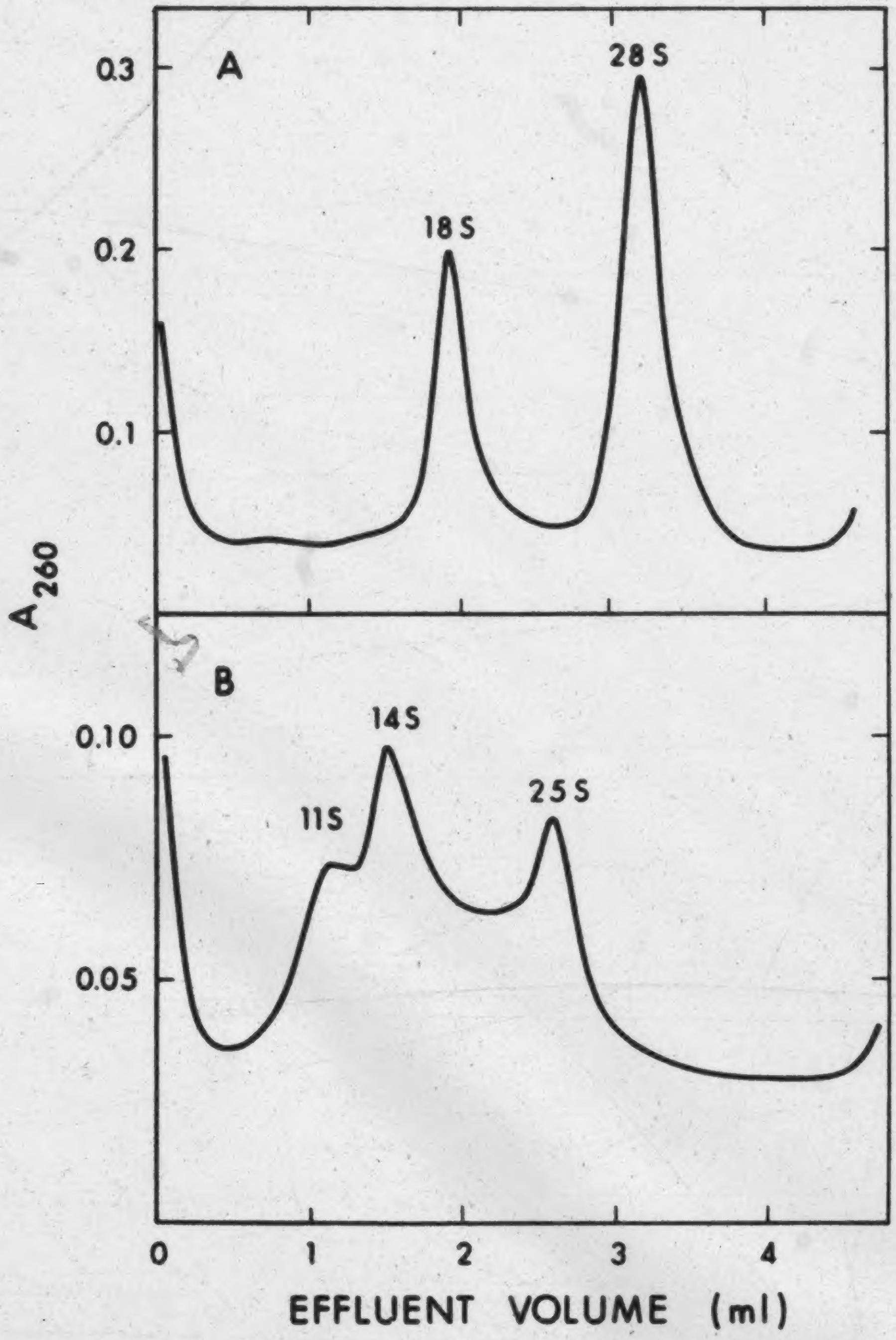


Figure 28. Sucrose density-gradient analysis of cellulose fractionated RNA. Linear sucrose gradients (10-20%) were made up with 20 mM Tris-HCl (pH 7.4), 1 mM Mg^{2+} and 50 mM NaCl. Centrifugation was in a Spinco SW 50.1 rotor at 48,000 rpm for 2.5 hr. A. Cellulose pass-through RNA. B. Cellulose-retained RNA.

Total polysomal RNA fractionated by cellulose chromatography into pass-through and retained fractions was assayed in the cell-free system for the ability to direct amino acid incorporation. Figure 29 shows [^{14}C]Leu incorporation as a function of the RNA concentration. Cellulose-retained RNA was found to have the highest incorporating activity (4-fold stimulation with this preparation of RNA). A relatively high incorporating activity was found in the cellulose pass-through fraction indicating that mRNA with low poly(A) content may be present in this fraction. Since the possibility exists that poly(A) may be sheared during phenol extraction (Perry *et al.*, 1972) an alternative method using SDS-EDTA for extraction of RNA followed by sucrose density-gradient fractionation was tried.

(d) SDS-EDTA extraction of polysomal RNA

Polysomes were disrupted with 1% SDS-5 mM EDTA and centrifuged through a linear 10-30% sucrose gradient containing SDS-EDTA (Rhoads *et al.*, 1972). The A_{260} sedimentation profile was nearly identical to that of Figure 26. Fractions corresponding to the 15-20S RNA were pooled and concentrated by ethanol precipitation to be used in the cell-free incorporating system for product analysis.

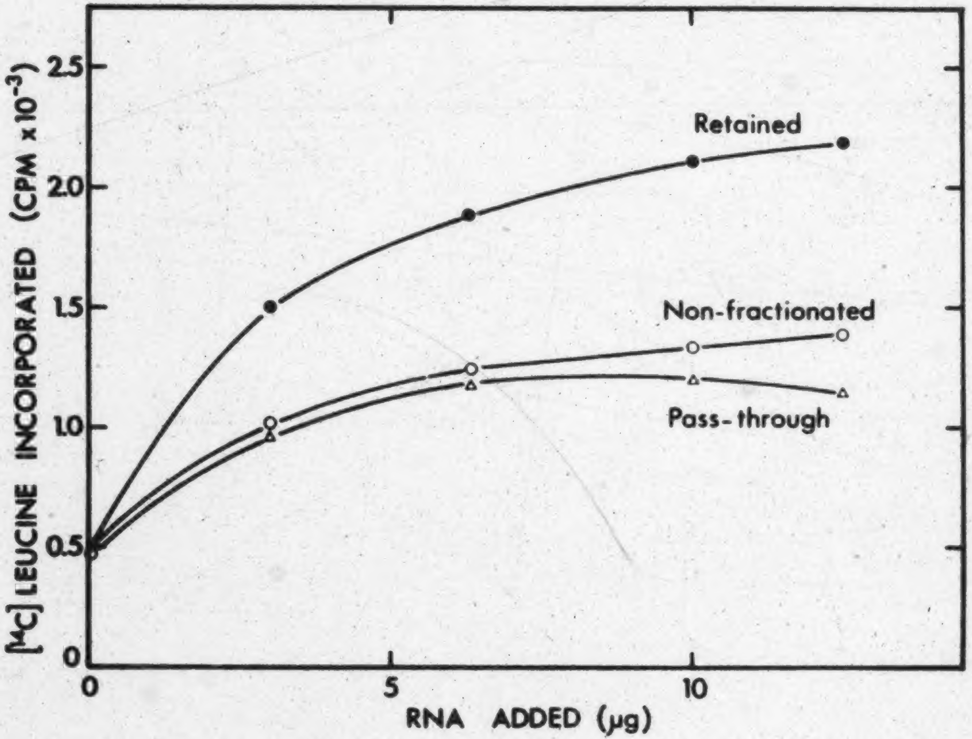


Figure 29. [^{14}C]Leu incorporation directed by RNA fractionated by cellulose column chromatography. The incorporation was carried out at 30° for 30 min at 3.5 mM Mg^{2+}, 65 mM KCl as described in Materials and Methods.

2. Translation of exogenous mouse liver mRNA in a cell-free system

(a) Some general properties of the cell-free system

Figure 30 shows the time course of ^{14}C-labeled amino acid incorporation into hot TCA-insoluble material in the presence of cellulose-retained RNA extracted from total polysomes. It is seen that the activity was dependent on added mRNA (Fig. 30A) and 0.5 M KCl ribosome wash (Fig. 30B). The rate of incorporation was linear over the time period 15 to 45 min, hence 30 min was selected as the incubation time for further experiments.

The Mg^{2+} concentration curve exhibited a narrow range for incorporation with the optimum occurring at 3.5 mM Mg^{2+} (Fig. 31A). Similar values have been reported in an ascites cell-free system for the translation of mRNAs, such as mouse globin and calf lens mRNA (2.5 mM Mg^{2+}, Mathews *et al.*, 1971; Mathews *et al.*, 1972), histone mRNA (3 mM Mg^{2+}, Jacobs-Loren, Baglioni, 1972) and mouse immunoglobin light-chain mRNA (3.7 mM Mg^{2+}, Schechter, 1973). The concentration of KCl was found to be less stringent than Mg^{2+} with the optimum at 65 mM KCl (Fig. 31B).

The effect of various concentrations of mRNA and ribosome wash on amino acid incorporation are shown in Figures 32 and 33, respectively. The relationship between rate of incorporation and RNA concentration was linear up to 7.5 µg RNA and reached a plateau at about 15 µg (Fig. 32) where a 9-fold stimulation of incorporation was observed.

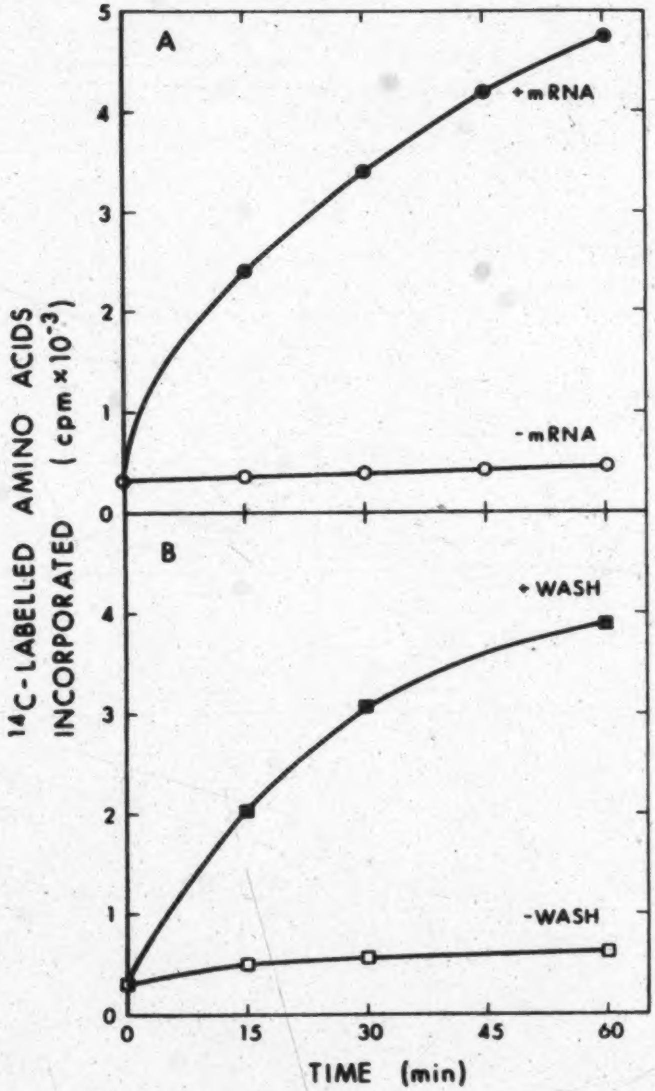


Figure 30. Time course of amino acid incorporation in the presence and absence of liver mRNA and ribosome wash. RNA was extracted from total polysomes, and fractionated by cellulose chromatography. The cellulose-retained RNA was used at a concentration of 10 μg per assay. The reaction mixture was incubated at 30^{0} and at the indicated times 100 μl sample was withdrawn and hot TCA-precipitable radioactivity was assayed as described in Materials and Methods. Background activity in the absence of ribosomes, 310 cpm was subtracted from each sample.

A. ●—●, with mRNA;○-○, without mRNA

B. ■—■, with ribosome wash;□-□, without ribosome wash.

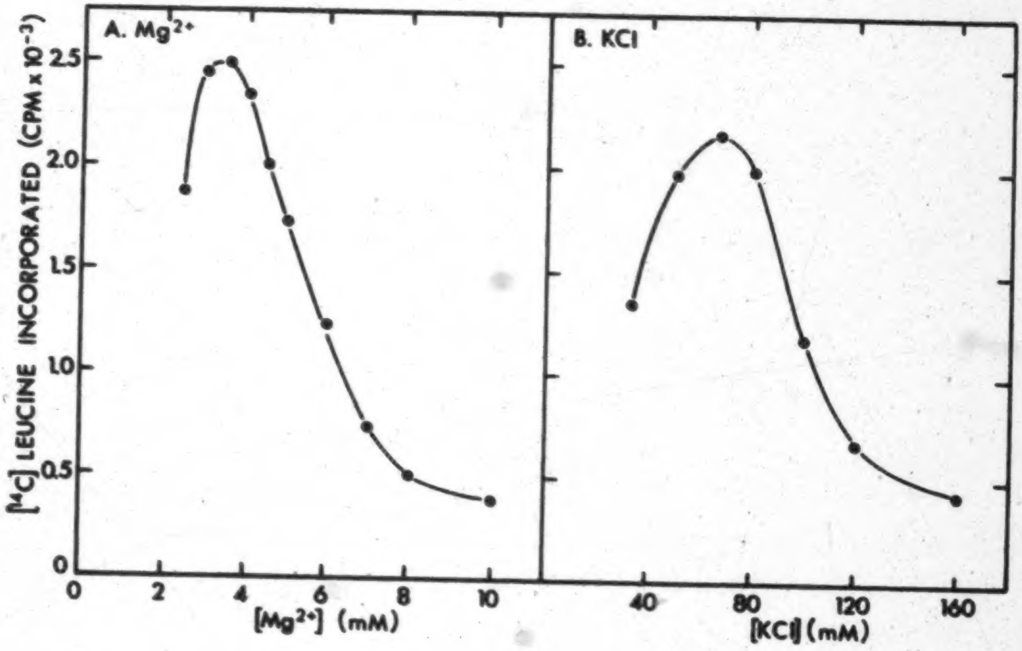


Figure 31. [^{14}C]Leu incorporation as a function of the Mg^{2+} and KCl concentration. RNA was extracted from total polysomes, was fractionated by cellulose chromatography, and the column-retained RNA was used at a concentration of 12 µg per assay. The incorporation was carried out at 30° for 30 min in the presence of 50 mM KCl (A.) and 3.5 mM Mg^{2+} (B.) as described in Materials and Methods.

A. Mg^{2+} concentration curve

B. KCl concentration curve

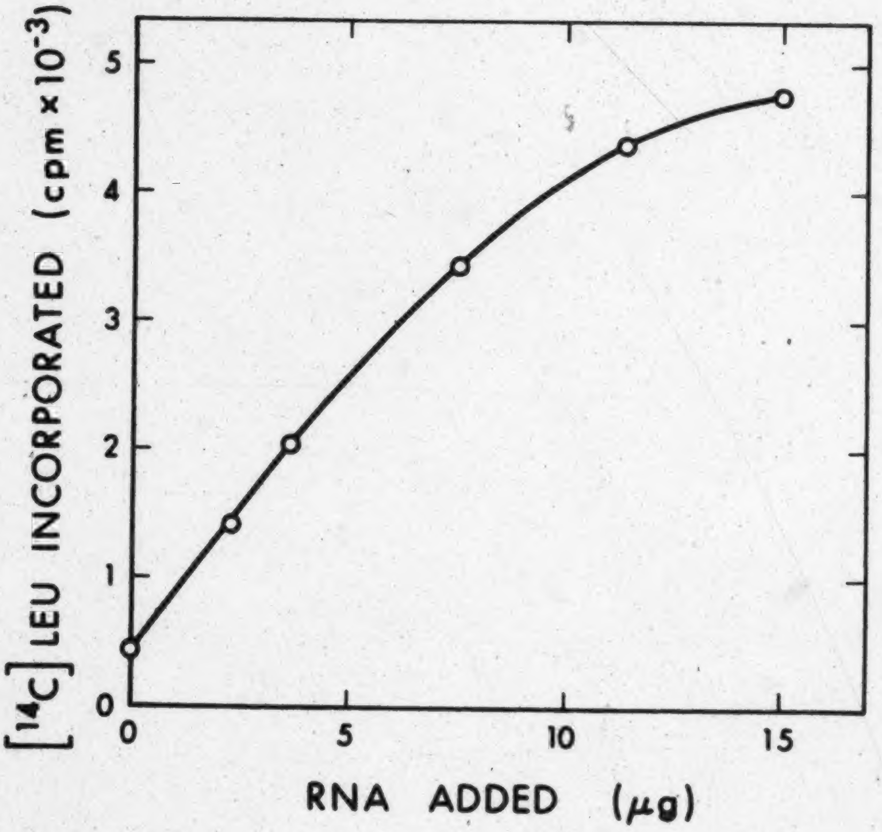


Figure 32. [^{14}C]Leu incorporation as a function of mRNA concentration. RNA extracted from total polysomes by the SDS-EDTA method was fractionated on a sucrose density-gradient containing 1% SDS - 5 mM EDTA, and the 15-20S RNA used in the cell-free amino acid incorporation system as described in the Materials and Methods. Incorporation was carried out at 30° for 30 min in the presence of 3.5 mM Mg^{2+}, 65 mM KCl and 150 μg ribosome wash protein. The background activity of 300 cpm was subtracted from each sample.

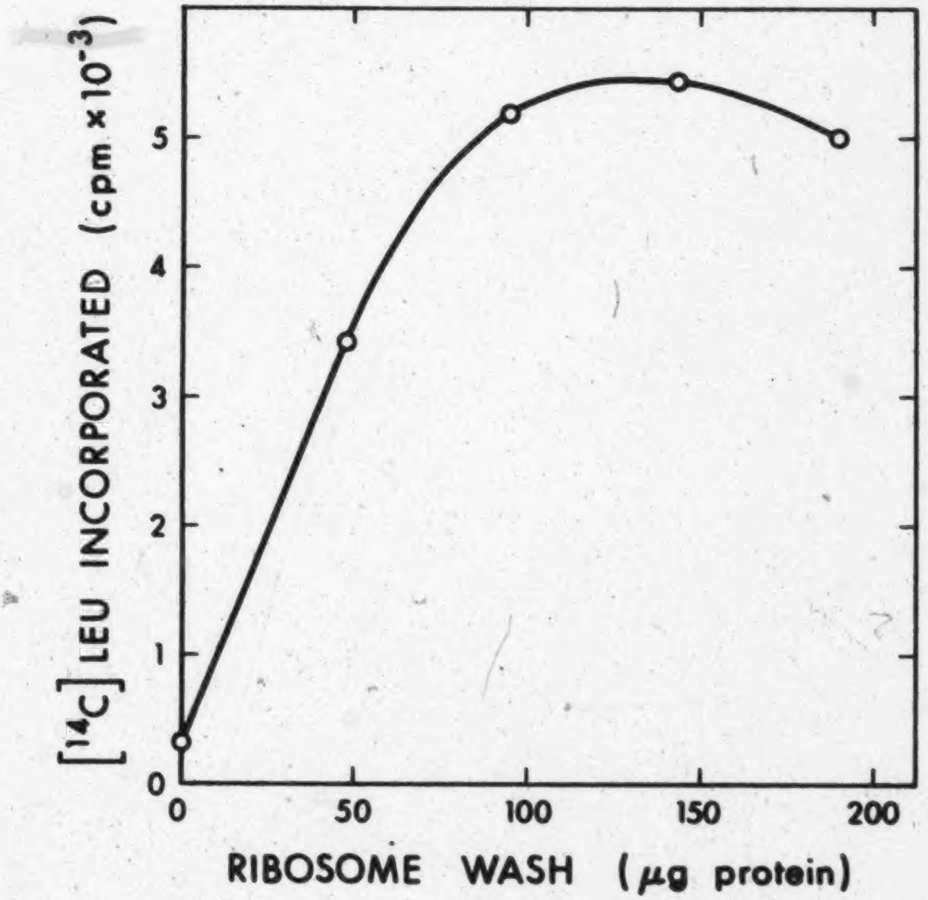


Figure 33. The effect of ribosome wash concentration on [^{14}C]Leu incorporation. The incorporation directed by 15-20S RNA was carried out as described in Fig. 32. 15-20S RNA was present at a concentration of 15 μg per assay.

Ribosome wash became saturating at 125 μg protein (Fig. 33), an amount equivalent to that needed for poly(U)-directed Phe incorporation as described in Chapter III.

Slight differences of incorporation level were observed with new preparations of polysomal RNA and ribosome wash, hence, for each preparation the optimum concentration was determined. The cellulose-retained RNA and 15-20S RNA were found to have the same Mg^{2+}, KCl, and ribosome wash optima. In some cases the specific activity of cellulose-retained RNA was higher than that of 15-20S RNA.

Amino acid incorporation directed by exogenous mRNA was examined as a function of run-off 80S ribosome concentration (Fig. 34). The total counts increased with the increasing concentration of ribosomes, but the counts incorporated per unit of A_{260} ribosome steadily declined. The highest specific activity was found at 2 A_{260} units/ml reaction mixture (0.2 A_{260}/assay). Similar results were obtained when examining the endogenous incorporation by liver polysomes (Fig. 35). Reasons for this apparent decline in activity will be discussed later.

(b) Polysome formation

In a cell-free amino acid incorporating system utilizing 80S ribosomes and exogenous mRNA, protein synthesis should be accompanied by the formation of polysomes. This was tested by analyzing the reaction mixtures in sucrose density-gradients. [^{14}C]Leu was included in the

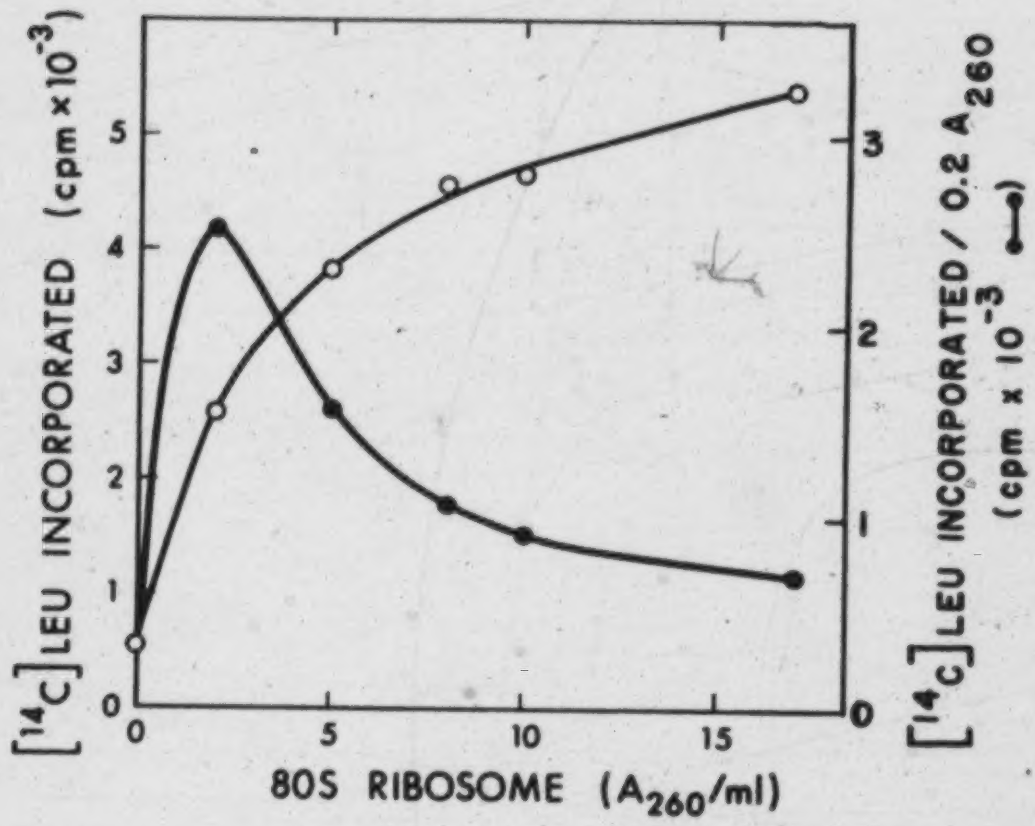


Figure 34. [^{14}C]Leu incorporation directed by exogenous mRNA as a function of 80S ribosome concentration. The incorporation directed by 15-20S RNA was carried out as described in Fig. 32. 15-20S RNA was present at a concentration of 15 μg per assay.

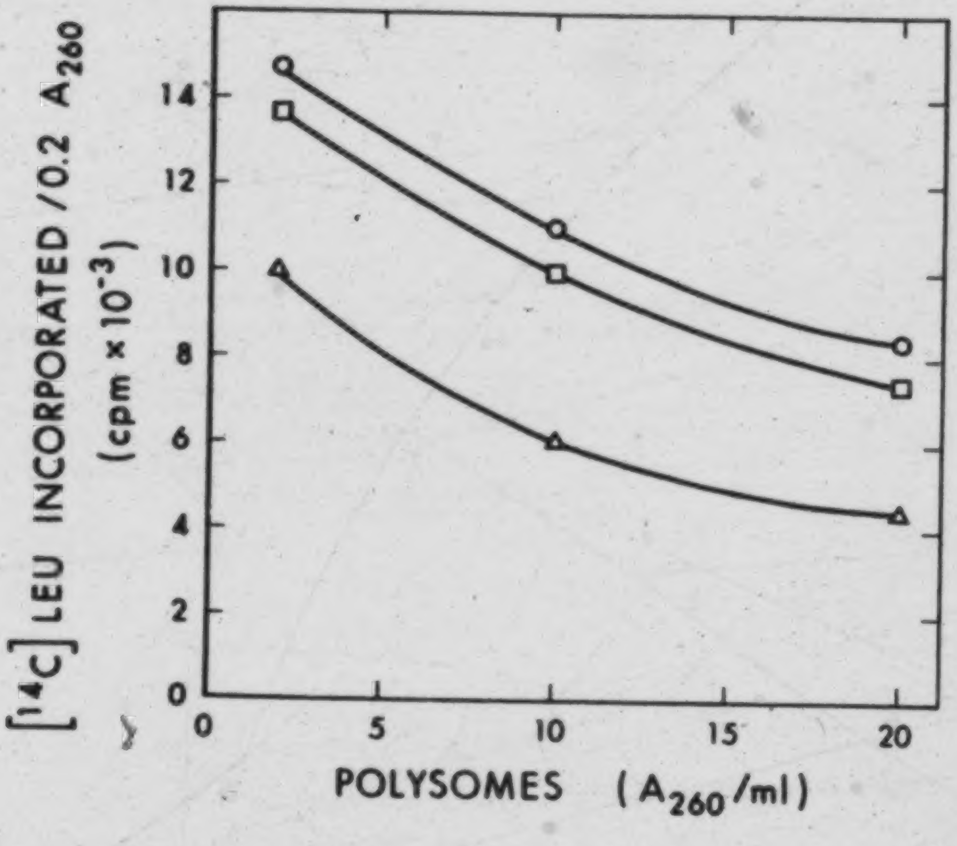


Figure 35. The effect of polysome concentration on endogenous incorporation. $[^{14}C]$Leu incorporation was carried out at 37° in the presence of 6 mM Mg^{2+} and 100 mM KCl in three reaction mixtures which contained 2 A_{260}/ml, 10 A_{260}/ml or 20 A_{260}/ml liver polysomes. At the indicated times, 100 µl samples were removed and hot TCA-precipitable radioactivity was determined as described in Materials and Methods.

△—△, incubated 10 min

□—□, incubated 30 min

○—○, incubated 60 min

reaction mixture to follow the distribution of newly-formed proteins in the gradients. Figure 36 shows the results of this experiment. In the absence of added mRNA (incubated at 30° for 30 min), some counts were observed on the top of the gradient but no radioactive material sedimented as polysomes (Fig. 36A). A sample containing both mRNA and ribosome wash but incubated at 0° also showed no formation of polysomes (Fig. 36B). However one incubated at 30° for 10 min did form radioactive material which sedimented in the region of polysomes containing 4-9 80S ribosomes (Fig. 36C). Continuation of incubation for 30 min resulted in a slight shift of the polysome peak to the lighter side and an increase of radioactivity on top of the gradient. These counts may represent released polypeptides.

This constitutes the first time, to the knowledge of the author, that exogenous mammalian mRNA has been shown to support the formation of polysomes in a cell-free amino acid incorporation system. Palacios and Schimke (1973) were able to synthesize ovalbumin using hen oviduct polysomal RNA in a reticulocyte lysate cell-free system. Sucrose density-gradient analysis of the reaction products was attempted, but the labeled polysome patterns were difficult to interpret due to the simultaneous endogenous incorporation by reticulocyte polysomes. Wang, Nasco and Arlinghaus (1972) have shown the ^{3}H-labeled Raucher leukemia viral RNA binds to ribosomes with the sedimentation pro-

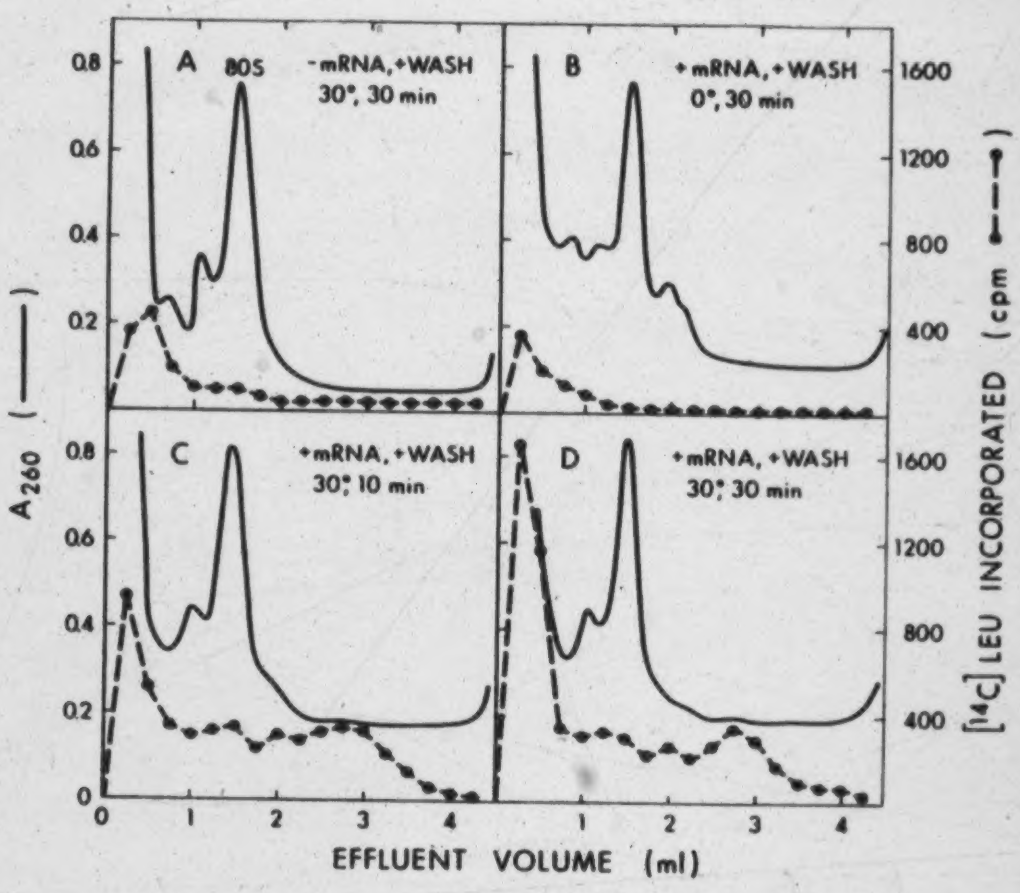


Figure 36. Sucrose density-gradient analysis of newly formed polysomes in the cell-free system translating exogenous mRNA. The complete reaction mixture (100 µl) contained 1 A_{260} run-off 80S ribosomes, 12 µg cellulose-retained RNA, 150 µg ribosomal wash protein, 3.5 mM Mg^{2+}, 65 mM KCl and other components as described in Materials and Methods. Convex-exponential sucrose gradients (0.3-1.0 M) were made up in 20 mM Tris-HCl (pH 7.4), 5 mM Mg^{2+} and 100 mM KCl. Centrifugation was in a Spinco SW 50.1 rotor at 40,000 rpm for 30 min. The gradients were fractionated and hot TCA-insoluble radioactivity determined as described previously.

A. Without mRNA, incubated at 30° for 30 min.
B. With mRNA, incubated at 0° for 30 min.
C. With mRNA, incubated at 30° for 10 min.
D. With mRNA, incubated at 30° for 30 min.

perties of polysomes when incubated in a cell-free system.

(c) Translation of exogenous liver mRNA by ribosome subunits.

Mouse liver ribosome subunits isolated by the method described in Chapter II supported exogenous mRNA-directed amino acid incorporation. A study on the kinetics of incorporation revealed that the 40S subunits alone showed no activity but when the 60S subunits were added a rapid increase in incorporation was observed (Fig. 37). The kinetics of this incorporation by the subunits was similar to that by 80S ribosomes (Fig. 30A).

Table 14 shows the results of an experiment in which the ability of our ribosome subunits to translate both synthetic and natural mRNA was tested. Poly(U)-directed Phe incorporation by run-off 80S and 40S + 60S ribosomes was as good as that reported in Chapter III. However, to obtain a Phe incorporation level equivalent to the 80S ribosomes,a 40S/60S A_{260} ratio of 1/1 was necessary. Hence, the 40S subunits were not as active as earlier preparations. When exogenous liver mRNA was used as template no incorporation was observed with the 40S subunits, while the 60S subunits incorporated [^{14}C]Leu equivalent to 5% of the 80S contraol. Mixtures of the 40S and 60S subunits in A_{260} ratios of 1/2 and 1/1 showed activity equivalent to 65% and 76% of the 80S control

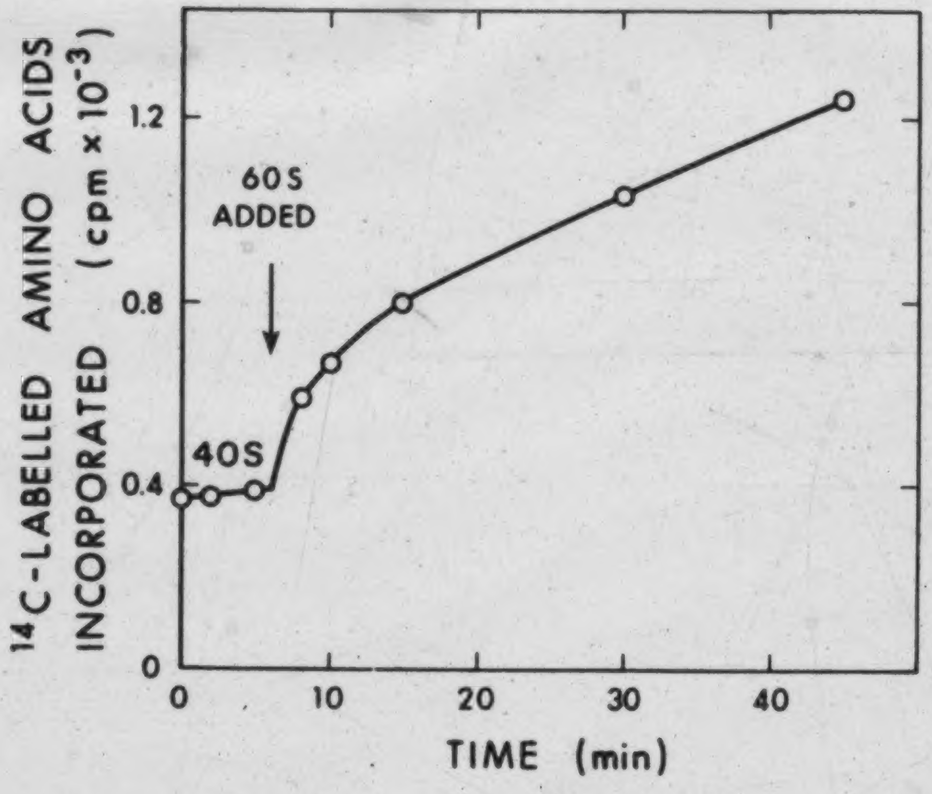


Figure 37. Time course of amino acid incorporation by ribosome subunits. Incorporation was carried out using ^{14}C-labeled amino acids at 30°. At the indicated times 100 µl samples were removed, which contained 50 µg 15-20S RNA (extracted from total polysomes), 0.06 A_{260} 40S ribosome subunits and other components as described in Materials and Methods. At 6 min, 60S subunits were added to the reaction mixture resulting in a 40S/60S A_{260} ratio of 1/2.5 (0.06 A_{260} 40S/0.15 A_{260} 60S per assay). The background activity was not subtracted.

Table 14. Translation of synthetic and natural mRNA by mouse liver ribosome subunits.

	Poly(U)-directed [^{14}C]Phe incorporation		Exogenous mRNA-directed [^{14}C]Leu incorporation	
Ribosomes	pmoles/A_{260}	% control	pmoles/A_{260}	% control
80S	154.7	100.0	11.7	100.0
40S	4.3	2.8	0.0	0.0
60S	2.1	1.4	0.6	5.1
40S + 60S[†]	134.6	87.0	7.6	65.0
40S + 60S[∫]	158.3	102.3	8.9	76.0

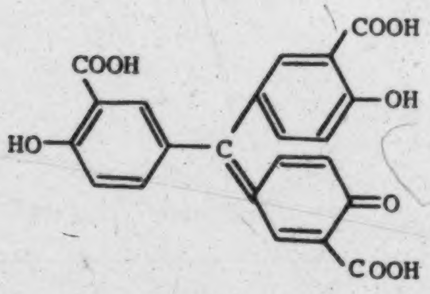
Incorporation was carried out at 30^{0} for 30 min as described in the Methods with each assay containing 0.2 A_{260} unit run-off 80S or 40S + 60S ribosomes, 40 µg poly(U) or 50 µg SDS-EDTA extracted 15 - 20S RNA, and 150 µg 0.5 M KCl ribosome wash. Phenylalanine incorporation was at 4 mM Mg^{2+}, 100 mM KCl while leucine was at 3.5 mM Mg^{2+}, 65 mM KCl. Background counts have been subtracted, 10 pmoles for poly(U) and 3 pmoles for mRNA-directed incorporation. † 40S/60S ratio 1/2; ∫ 40S/60S ratio 1/1.

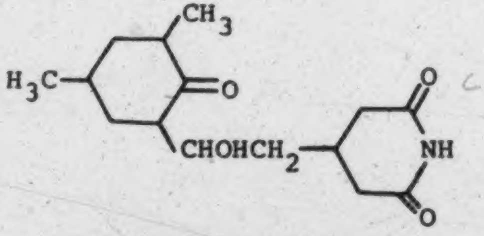
value, respectively.

The level of Phe incorporation directed by poly(U) with 80S ribosomes was excellent indicating the IF-M_1 and IF-M_2 in the ribosome wash were functional (Shafritz, Anderson 1971). On the other hand the translation of exogenous mRNA was relatively poor (11.7 pmoles [^{14}C]Leu/A_{260} ribosome as compared to 47.6 pmoles/A_{260} ribosome in Fig. 32 and 33). This suggests that either mRNA or messenger-specific initiation factor in the ribosome wash (IF-M_3)may have been partially inactivated.

(d) Inhibition studies: aurintricarboxylic acid and cycloheximide

Antibiotics have been used with some success in identifying the sequence of reactions that occur on the ribosome during protein synthesis. Aurintricarboxylic acid (ATA, ammonium salt known as aluminon) and cycloheximide (actidione) are among those antibiotics that inhibit mammalian protein synthesis. The chemical structure for each is given below.


Aurintricarboxylic acid


Cycloheximide

Low concentrations of ATA ($<10^{-4}$ M) inhibited the binding of f_2 and Q_β mRNA to *E. coli* ribosomes (Grollman, Stewart, 1968), globin mRNA to rabbit reticulocyte 40S subunits (Lebleu *et al.*, 1970), and at much higher concentrations, peptide chain elongation and termination (Webster, Zinder, 1969). Cycloheximide binds to the 60S ribosome subunit and therefore only inhibits mammalian protein synthesis (Rao, Grollman, 1967). This inhibition was primarily due to blockage of chain elongation by inactivation of elongation factor EF_2 (Godchauz *et al.*, 1967; Baliga *et al.*, 1969; Baliga *et al.*, 1970), and interference with release of deacylated tRNA from the donor site (McKeehan, Hardesty, 1969; Obrig *et al.*, 1971). Cycloheximide also interferes with the process of initiation at concentrations lower than that which inhibits elongation (Munro *et al.*, 1968).

Effect of various concentrations of aurintricarboxylic acid and cycloheximide on the translation of endogenous mRNA using liver polysomes and of exogenous mRNA using 80S ribosomes were tested. Logically one would expect that peptide chain elongation is the primary event in the translation of endogenous mRNA while peptide chain initiation is a prerequisite for the translation of the extracted mRNA, and that these differences could be detected by the inhibitors. Translation of exogenous mRNA should be more sensitive to a low concentration of aurintricarboxylic acid. Figure 38 shows the inhibition curves obtained with aurintricarboxylic acid. To achieve a 50% inhibition of endogenous protein synthesis, 2×10^{-3} M aurintricarboxylic acid was required as compared to 4×10^{-5} M for exogenous incorporation. This represents a 200-fold difference in sensitivity. Moreover, at 1×10^{-5} M aurintricarboxylic acid, exogenous mRNA-directed incorporation was inhibited 80% whereas no effect was observed on protein synthesis by polysomes. A much less significant different of inhibition was found with cyclohexide (Fig. 39). The inhibition curves were nearly parallel with 50% inhibition occurring at 2×10^{-4} M cycloheximide for the endogenous, and 2×10^{-5} M for the exogenous incorporation. It is clear from these results that in the presence of added mRNA, the protein synthesis observed includes peptide chain initiation as well as peptide elongation.

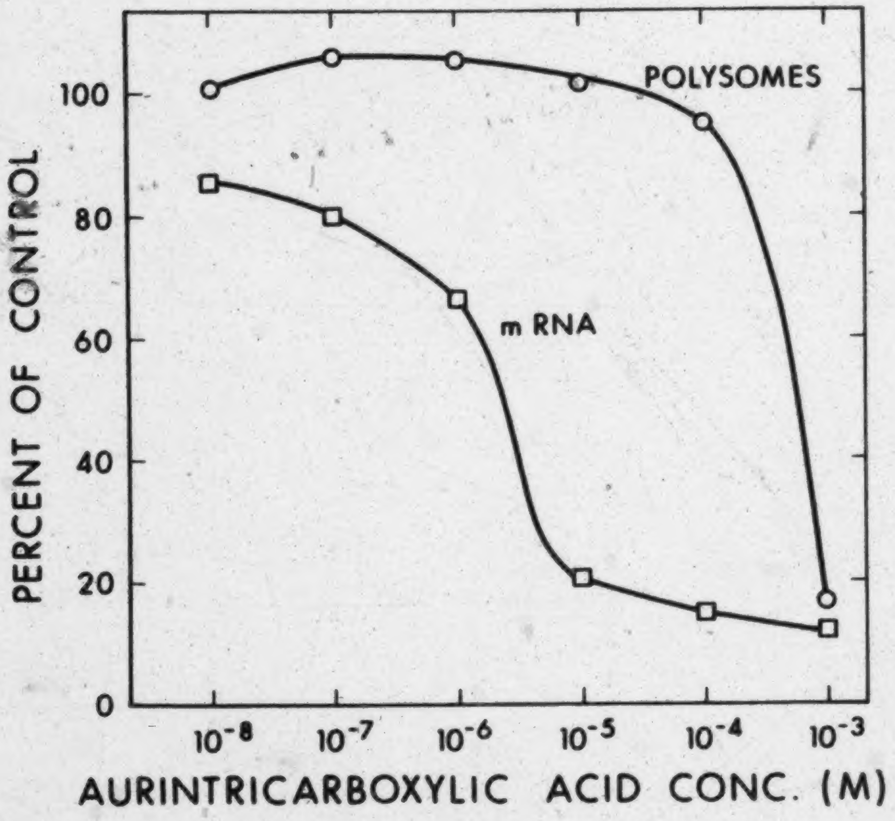


Figure 38. Inhibition of translation of endogenous and exogenous mRNA by aurintricarboxylic acid. $[^{14}C]$Leu incorporation was carried out at 30° for 30 min as described in Materials and Methods. Exogenous mRNA (15-20S RNA) was present at 50 μg per assay. Total polysomes were present at 0.2 A_{260} unit per assay. Aurintricarboxylic acid was dissolved in water and added to each reaction mixture before the 15-20S RNA or polysomes. Control represented the amount of incorporation observed in the absence of the antibiotic.

□—□, translation of exogenous mRNA

○—○, translation of endogenous mRNA

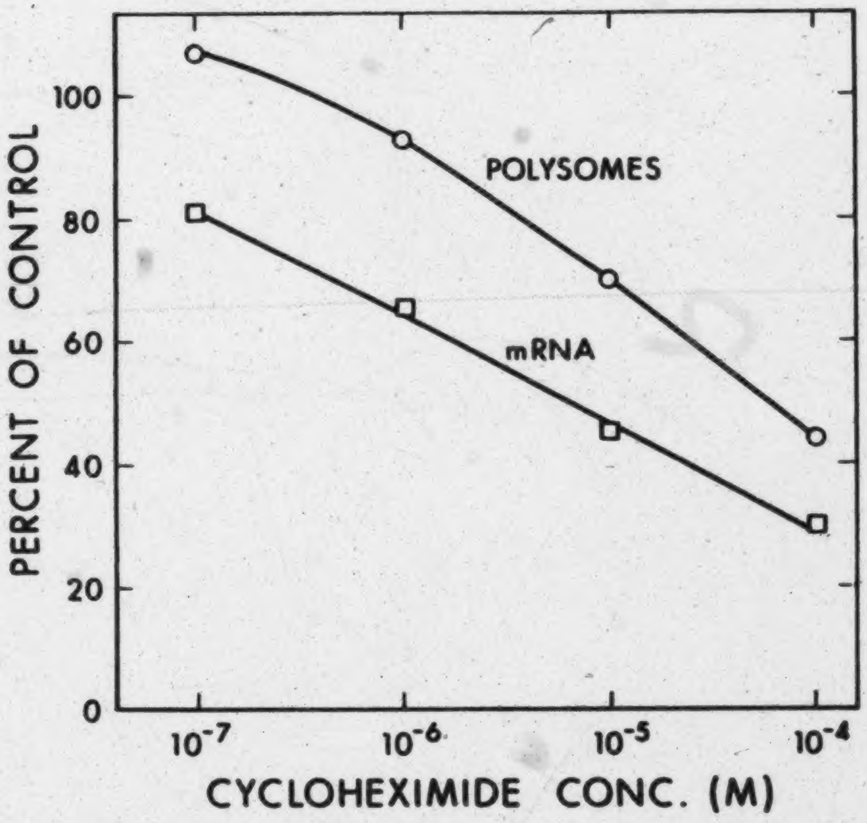


Figure 39. Inhibition of translation of endogenous and exogenous mRNA by cycloheximide. [^{14}C]Leu incorporation was carried out as described in Figure 38. Cycloheximide was dissolved in water and added to each reaction mixture before the 15-20S RNA or polysomes. Control represented the amount of incorporation observed in the absence of the antibiotic.

□—□, translation of exogenous mRNA

○—○, translation of endogenous mRNA

3. Product analysis: identification of albumin

(a) Large scale incorporation for product analysis

Evidence presented in this Chapter to this point indicates that mouse liver mRNA has been isolated and translated in our cell-free incorporation system. However, conclusive evidence to support this argument would be to show synthesis of a particular protein. Liver is a complex tissue in which a wide array of proteins for intracellular and extracellular use are synthesized. Albumin was chosen as the protein to be studied because it represents a large portion of the protein synthesized by liver and it is readily identifiable by electrophoresis due to its high negative charge.

For product analysis a reaction mixture of 1 to 5 ml was incubated at 30° for 60 min. [^{14}C]Leu was replaced with a mixture of 15 ^{14}C-labeled amino acids supplemented with the remaining 5 cold amino acids. Table 16 summarizes data typical of a large scale incorporation of labeled amino acids by membrane-bound polysomes, total polysomes (treated with detergent) and run-off 80S ribosomes in the presence of extracted mRNA. 50-70% of the radioactive material synthesized by total polysomes and extracted mRNA was released directly into the supernatant. However, only 14.5% of the labeled polypeptides produced by membrane-bound polysomes were released into the supernatant. This indicated that the major part of these

Table 15. Large scale amino acid incorporation by mouse liver polysomes and extracted polysomal RNA.

Sample	Endogenous incorporation by polysomes (pmoles/A_{260})				Exogenous incorporation by extracted mRNA (pmoles/A_{260})			
	Membrane-bound polysomes	% release	Total polysomes	% release	SDS-phenol	% release	SDS-EDTA	% release
Incubation, 30°, 60 min	28.8	-	41.4	-	24.6	-	38.4	-
Post-ribosomal supernatant	4.2	14.5	23.8	57.5	18.3	74.5	22.7	59.2

[^{14}C]Leu incorporation in a total volume of 1 ml was carried out at 30° for 60 min as described in Materials and Methods. After incubation ribosomes were removed from the reaction mixture by centrifugation in a Spinco Type 50 rotor in 2 ml adaptor tubes at 149,000 x g (av) for 90 min. Hot TCA-insoluble radioactivity was determined before and after centrifugation.

newly-synthesized proteins entered the reticulum cisternae as required for extracellular transport. Disruption of the membranes by freeze-thawing and sonication enabled recovery of 50% of the labeled membrane-associated polypeptides. Albumin synthesized on the membrane-bound polysomes and recovered in this way was used as a control in the immunological and gel analysis of products synthesized by exogenous mRNA.

(b) Identification of albumin by polyacrylamide gel electrophoresis and immunoprecipitation

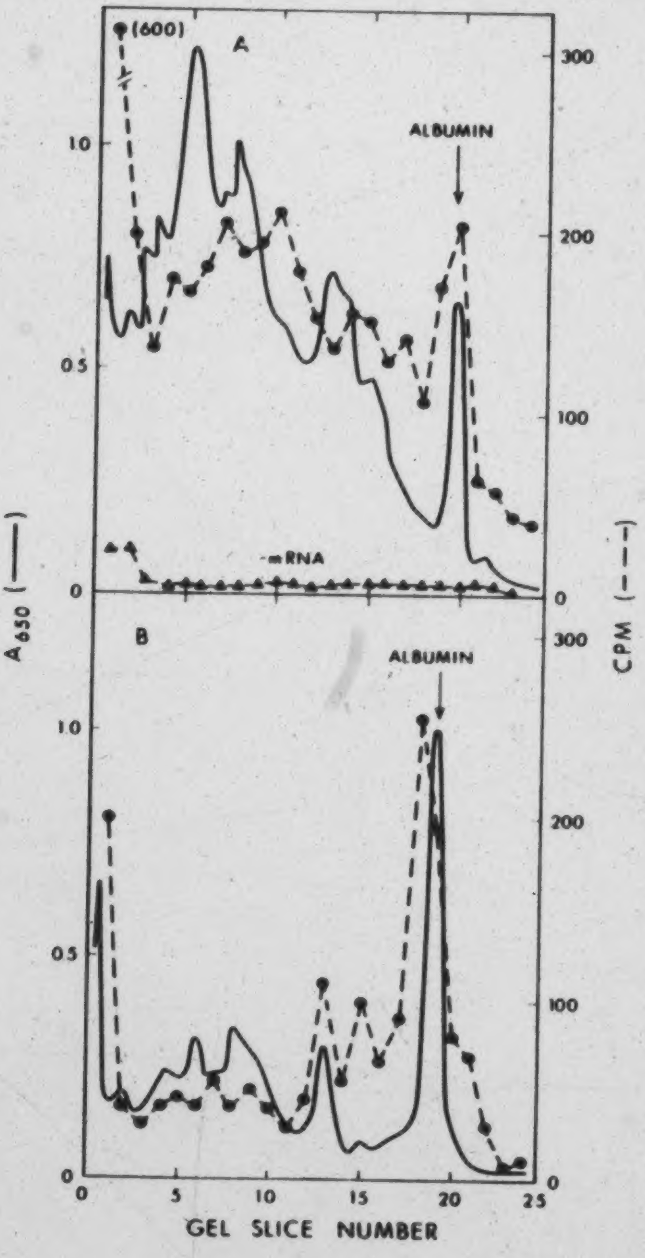
When the products obtained with mRNA extracted from membrane-bound polysomes were analyzed by polyacrylamide gel electrophoresis, about 8% of the total radioactivity was found to be coincident with the albumin fraction (Fig. 40A). A control sample incubated in the absence of polysomal RNA showed little radioactivity throughout the gel. Gel analysis of the products of endogenous protein synthesis by membrane-bound polysomes is presented in Figure 40B. About 25% of the total radioactivity recovered from the gel was in the albumin fraction. This figure, however, is based on radioactivity released from the microsomal membranes and cisternae by freeze-thawing and sonication, and should not be directly compared with the percent of albumin synthesis represented in Figure 40A.

Figure 40. Polyacrylamide gel analysis of cell-free products directed by exogenous and endogenous mRNA. Electrophoresis was carried out in 7% gel at pH 8.9 as described in Materials and Methods.

A. Proteins produced by mRNA extracted from membrane-bound polysomes. The components of the reaction mixture were the same as described in Materials and Methods except that the total volume was 1 ml. After incubation at 30°C for 60 min the sample was centrifuged at 149,000 x g (av) for 90 min and the supernatant was analyzed in gel.

●--● , with mRNA; ▲--▲ , without mRNA.

B. Products of endogenous protein synthesis by membrane-bound polysomes. Membrane-bound polysomes (2 A_{260} units) were incubated with 0.5 μCi [^{14}C]Leu, with components as described above for 60 min. The reaction mixture was centrifuged at 149,000 x g (av) for 90 min, the pellet was suspended in water, frozen and thawed and then sonically disrupted for 5 min in a Bronson Sonifier at setting number 5. This was then centrifuged at 149,000 x g (av) for 90 min and the supernatant analyzed by polyacrylamide gel electrophoresis.


(600)
A
ALBUMIN
-mRNA
B
ALBUMIN
A_{650} (——)
CPM (- - -)
1.0
0.5
0
300
200
100
0
0
5
10
15
20
25
GEL SLICE NUMBER

The amount of albumin synthesized as determined by gel electrophoresis was highly reproducible, with several RNA preparations from membrane-bound polysomes it ranged from 5 to 8%, and with RNA from total polysomes, 3 to 4% of the total counts on the gel. Further fractionation of polysomal RNA by cellulose column chromatography, or by using the 15-20S RNA fractions after sucrose density-gradient centrifugation, resulted in an overall increase in specific activity but not in apparent enrichment of albumin mRNA. Figure 41 shows the gel analysis of the products synthesized by these two RNA preparations.

Radioactive albumin in the cell-free products was also analyzed by precipitation with anti-albumin serum (Table 16). The results were in agreement with those obtained by gel electrophoresis although in several analysis the immunological assay tended to give slightly higher values of albumin synthesis than gel analysis. Before precipitation with anti-albumin serum the reaction mixtures were cleared of nonspecific precipitable material by the addition of chicken serum and rabbit antiserum to chicken serum. These precipitates usually contained 100 cpm to 150 cpm, and if the clearing reaction was repeated a second time, low counts (<50 cpm) were found in these precipitates. It is still possible that non-specific precipitation occurs with anti-albumin serum and, in addition, precipitation of incomplete albumin chains (Hill *et al.*, 1972; Uenoyama, Ono, 1972; Taylor, Schimke, 1973) may contribute to higher values of albumin synthesis.

In order to further confirm that the radioactivity in the albumin fraction of the gel represented newly-synthesized albumin, we subjected the cell-free products to a purification procedure (as described in Materials and Methods) with respect to albumin and then analyzed by gel electrophoresis. The radioactivity in the albumin fraction before (Fig. 42A) and after purification (Fig. 42B) was 4% and 19%, respectively, of the total counts on the gel. This increase in radioactivity corresponded with the degree of purification of albumin (approximately 4-fold) in the sample indicating that the radioactivity in the albumin fraction in fact reflected the newly-synthesized albumin.

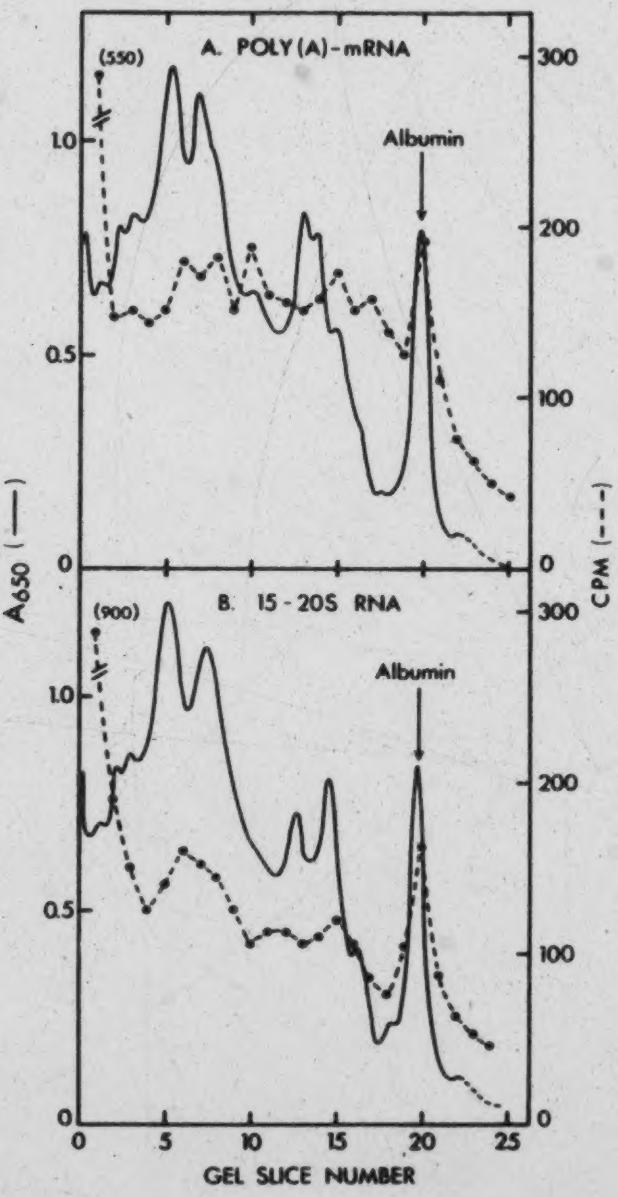


Figure 41. Polyacrylamide gel analysis of cell-free products directed by polysomal RNA. Conditions for incorporation and gel analysis were as described in Figure 40A and in Materials and Methods. Poly(A)-containing (cellulose-retained) RNA and 15-20S RNA from total polysomes were present at 10 μg and 50 μg per 0.1 ml reaction mixture, respectively.

A. Proteins produced by poly(A)-containing RNA.

B. Proteins produced by 15-20S RNA.

Table 16. Precipitation of cell-free products by rabbit antiserum to mouse albumin.

	Endogenous				Extracted mRNA			
	Membrane-bound polysomes		Total polysomes		Membrane-bound polysomal RNA		Total polysomal RNA	
	cpm	%	cpm	%	cpm	%	cpm	%
Total radioactivity	3300	-	4500	-	4225	-	5500	-
Radioactivity precipitated	980	30.0	820	18.0	255	6.0	210	3.8

Samples used for analyses were prepared according to the methods described in the legend of Fig. 40A for extracted mRNA and Fig. 40B for endogenous mRNA. In order to remove non-specific background, 150 µl samples were first incubated with chicken serum and rabbit antiserum to chicken serum at 37° for 60 min (Redman, 1969). The precipitates were removed by centrifugation, and to the supernatants mouse serum and rabbit antiserum to mouse albumin (Nutritional Biochemicals Corporation, Cleveland, Ohio) were added and incubated at 37° for 60 min, and then overnight at 4°C. The precipitate was washed three times with 10 mM Tris-HCl (pH 7.8) - 150 mM NaCl, resuspended in 1 ml 10% TCA and collected on Millipore filters for counting in a liquid scintillation counter.

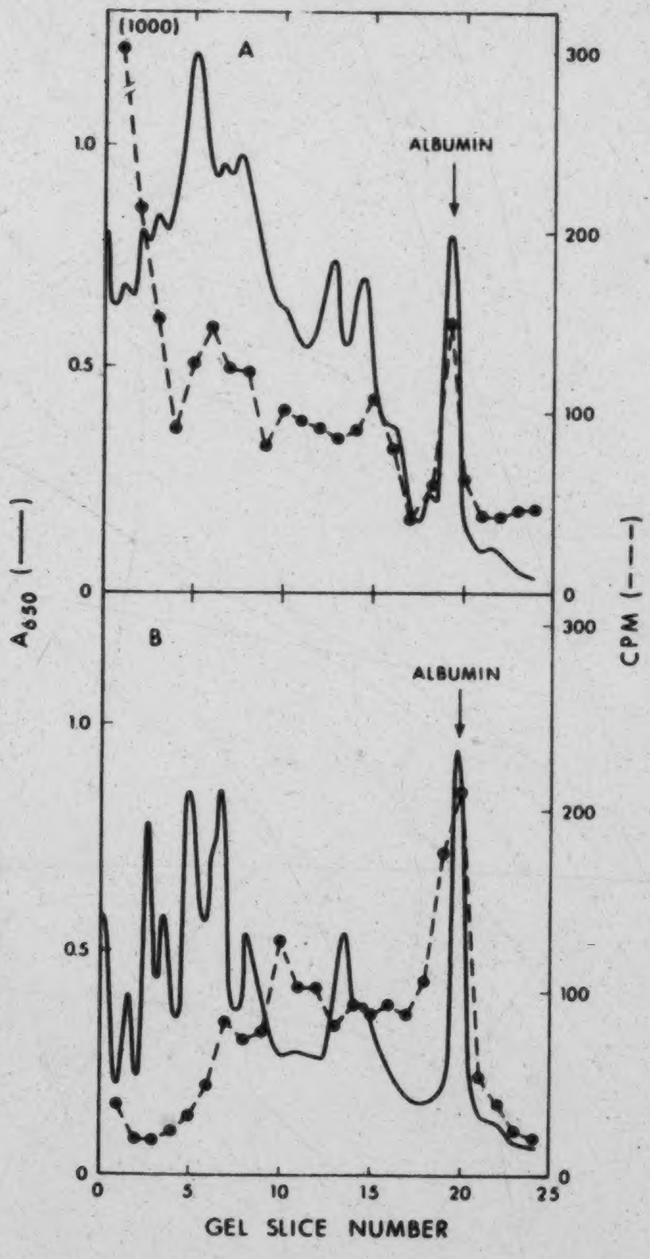


Figure 42. Polyacrylamide gel analysis of cell-free products before and after albumin purification. The experimental conditions were the same as described in Figure 40A except ^{14}C-protein hydrolysate was used. 15-20S RNA was present at a concentration of 50 μg per 0.1 ml reaction mixture.

A. Proteins produced by 15-20S RNA extracted from total polysomes

B. Same as A, but after partial purification of albumin

D. Discussion

The first mammalian protein synthesized by exogenous mRNA in a cell-free incorporation system was mouse reticulocyte β-globin chains (Lockard, Lingrel, 1969). This success can be partially attributed to the fact that reticulocytes synthesize essentially one class of proteins, α- and β-globin chains of hemoglobin, which are coded by monocistronic mRNAs with a sedimentation coefficient of 9S (Bulova, Burka, 1970). Since mRNA represents approximately 1% of the cellular RNA, the problem of its isolation involves separating it from the 18S and 28S ribosomal RNA. Fractionation of reticulocyte polysomal RNA by sucrose density-gradient centrifugation allows one to select the 9S peak, and by repeating the sucrose gradient fractionation, highly pure and active globin mRNA preparations have been obtained (Gurdon *et al.*, 1971; Housman *et al.*, 1971; Mathews *et al.*, 1971). Other mRNAs purified by sucrose gradient fractionation based on S values of polysomes and RNA extracted include lens α-crystallin (Mathews *et al.*, 1972), histone (Jacobs-Lorena *et al.*, 1972), myosin (Heywood, Nwagwu, 1969) and myoglobin (Thompson *et al.*, 1973).

Two other approaches to obtain an RNA preparation enriched with mRNA include the use of cellulose or cellulose derivatives to bind poly(A)-containing mRNA, and the selective immunoprecipitation of polysomes by antibody to the protein they synthesize. By these methods mRNA for catalase (Uenoyama, Ono, 1972), immunoglobin (Schechter, 1973), ovalbumin (Palacios *et al*, 1972) and tryptophan oxygenase (Schutz *et al*., 1973) have been isolated.

Isolation of mRNA for albumin presents a more difficult challenge than that for smaller proteins such as globin or myoglobin. Albumin is a stable, single polypeptide chain containing no other constituents with a molecular weight of 65,000 daltons (Peters, 1962). Messenger RNA coding for a protein of this size would contain about 1700 nucleotides, and assuming 200 adenine residues are present in the poly(A) segment, necessitates a molecule of molecular weight 5.5×10^5 daltons. The portion of mRNA coding for albumin would be approximately 5800 Å in length (1725 nucleotides x 3.4 Å). If a space of 300-350 Å exists from center to center of each translating ribosome, albumin synthesizing polysomes could contain 16-18 80S ribosomes. In comparison, globin or myoglobin polysomes would require 4-6 80S ribosomes (Temple, Housman, 1972; Thompson *et al*., 1973; Low, Rich, 1973).

It is clear from the results presented in this Chapter that the mouse liver cell-free incorporation system using run-off 80S ribosomes was suitable for the translation of exogenous mRNA. This system was completely dependent on added mRNA and ribosome wash for activity (Fig. 30). Ionic conditions were also found to play an important role in the translation of exogenous mRNA. In the case of Mg^{2+} amino acid incorporation was reduced by 50% when the Mg^{2+} concentration was changed to 1 mM below or 2 mM above the optimum of 3.5 mM Mg^{2+} (Fig. 31). Mathews (1972) observed that there were different salt optima for the translation of globin mRNA and EMC virus RNA in a Krebs II ascites cell-free system. He points out that Mg^{2+} and K^+ concentrations could be selected in which one messenger was translated efficiently while the other hardly at all.

Results presented in Figures 34 and 35 illustrate that factors other than ionic conditions can influence the size of proteins synthesized in a cell-free system. As the total concentration of run-off 80S ribosomes or polysomes increased, the amino acid incorporation/0.2 A_{260} unit was found to decrease. A large excess of ribosomes could possibly deplete some substrate causing premature termination of protein synthesis. Polyacrylamide gel analysis of polypeptides synthesized by various concentrations of polysomes has further substantiated this conclusion (Miall, Tamaoki, unpublished results).

In this study two different experiments confirmed that the observed amino acid incorporation in our cell-free system was due to peptide chain initiation and elongation. Newly-formed polysomes could be detected in the cell-free system by sucrose density-gradient analysis. Deletion of either mRNA or ribosome wash resulted in no polysome formation (Fig. 36). Secondly, the antibiotic, aurintricarboxylic acid, inhibited exogenous incorporation at a concentration which had no effect on the completion of growing polysomal polypeptides. Although the precise interaction of aurintricarboxylic acid with the "pre-initiation complex" is not known, most evidence supports the theory that it interferes with the binding of mRNA to the 40S ribosome subunit. Recently Ayuso-Parilla, Hirsch and Henshaw (1973) found that aurintricarboxylic acid inhibited the binding of specific proteins to the native 40S subunits. According to the ribosome cycle that they propose (Henshaw *et al.*,1973), these non-ribosomal proteins must bind to the 40S subunit before mRNA can be bound. It is not certain at this time whether these proteins are identical to the mammalian initiation factors. However, it is obvious when comparing the two sets of inhibition curves (Fig. 38 and 39), that aurintricarboxylic acid at low concentration (10^{-4} M) inhibited some part of the initiation process, whereas the effect of cycloheximide was more general affecting both peptide chain initiation and elongation.

It was found that albumin mRNA, like many other mammalian mRNAs, was bound to cellulose at high ionic strength and therefore probably contains a poly(A) segment. Sucrose density-gradient analyses of the retained RNA showed components with molecular weights estimated by Gierer's equation[1] (1958) to range from 2.1×10^5 to 1.3×10^6

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[1] Gierer's equation was based on measurements of TMV RNA made in a 0.02 M phosphate (pH 7.0) at 5^o; $M=1100\ S^{2.2}$ where M and S represent molecular weight and sedimentation coefficient, respectively.

daltons (McConkey, 1967). Although these values are only approximate (due to possible differences of hydrodynamic effects), they are in the range of the theoretical value for albumin mRNA.

The results of the immunoprecipitation and polyacrylamide gel analyses indicated that liver polysomal RNA isolated by either cellulose column chromatography or sucrose gradient fractionation, could direct the synthesis of albumin in the homologous cell-free system. That the observed incorporation truly represented newly synthesized albumin was further verified by its partial purification. The increase in counts in the albumin fraction after purification was in agreement with the 4-fold purification of albumin.

In most cases the 15-20S polysomal RNA from the sucrose gradients had a higher activity in the cell-free system than RNA isolated by cellulose column chromatography. This higher activity could be due to a protective effect by ribosomal RNA from ribonuclease degradation or that the mildness of the SDS-EDTA extraction procedure minimized damage to the mRNA. Since the percentages of albumin synthesized on cellulose-retained RNA and 15-20S RNA were similar, it is suggested that the difference was due to a general loss of activity rather than a specific loss of albumin mRNA. Possibly a better approach to the problem, knowing that both types of RNA code for albumin would be to treat polysomes with SDS-EDTA, isolate the 15-20S RNA from sucrose gradients and apply this to a cellulose column for final purification.

While in the process of writing this thesis two reports on the cell-free synthesis of albumin in heterologous systems came to our attention. Taylor and Schimke (1973) using rat liver total polysomal RNA were able to demonstrate the cell-free synthesis of albumin and found the highest albumin synthesizing activity in the 18S RNA peak. Shafritz (1974) found exogenous rabbit liver polysomal RNA capable of directing the synthesis of albumin in a reticulocyte cell-free system. Comparison of the proteins synthesized by free and membrane-bound polysomal RNA revealed albumin to be synthesized by both. These results suggest that albumin mRNA is present in free polysomes as well as membrane-bound polysomes, but in the case of the free polysomes it is in a non-translatable form. Once the RNA was extracted, however, the mechanism controlling this non-translation would be defeated thereby allowing albumin synthesis to occur in the cell-free system.

CHAPTER V

GENERAL CONCLUSIONS

From recent studies on the structure and function of ribosomes it is becoming increasingly apparent that they play a more direct role in the regulation of protein synthesis by processes which are independent of the rate of mRNA synthesis.

When the mRNA concept was first formulated, it was believed that ribosomes were inert scaffolds upon which mRNA attached to be translated. However, it is now known that the process of protein synthesis, and of initiation in particular, is complex requiring the presence of specific protein factors. It has been suggested that several classes of these "protein initiation factors" may exist to regulate the production of specific proteins (Thompson *et al.*, 1973). The function of this mechanism would be to allow a "fine tuning" of the post-transcriptional events of gene expression whereby proteins required for a particular phase of cell development would be synthesized in a synchronous manner.

With the advent of methods to isolate and translate natural mRNA in a cell-free incorporation system, it will be possible to study the synthesis of a specific protein under the influence of initiation factors isolated from cells at different stages of differentiation.

Current application in this laboratory of the cell-free systems described has allowed the study of several proteins: one of which is α-fetoprotein, an embryonic protein normally present in fetal liver. α-fetoprotein in adult serum, however, has been found to indicate the presence of a neoplasm (e.g. hepatoma). It appears that with the development of cancer, the embryonic genes are activated to either produce α-fetoprotein mRNA or if the mRNA is already synthesized, to somehow allow the messenger to be translated. A cell-free system utilizing exogenous mRNA will enable investigation of the possiblity of this being a transcriptional or translational control mechanism. It is hoped that the methods developed in this thesis will enable further studies to be done on the mechanism and control of mammalian protein synthesis.

BIBLIOGRAPHY

Adesnik, S.D., and Darnell, J.E., 1972. Biogenesis and characterization of histone messenger RNA in Hela cells. J. Mol. Biol. *67*:397.

Arlinghaus, R., Shaeffer, J., Bishop, J., and Schweet, R. 1968. Purification of the transfer enzymes from reticulocytes and properties of the transfer reaction. Arch. Biochem. Biophys. *135*:604.

Armstrong, J.A., Edmonds, M., Nakazoto, H., Phillips, B.A., and Vaughan, M.H. 1972. Polyadenylic acid sequences in the virion RNA of poliovirus and eastern equine encephalitis virus. Science *176* 526.

Ayuso-Parilla, M., Hirsch, C.A., and Henshaw, E.C. 1973. Release of the non-ribosomal proteins from the mammalian native 40S ribosomal subunit by aurintricarboxylic acid. J. Biol. Chem. *248*:4394.

Baliga, B.S., Cohen, S.A., and Munro, H.N. 1970. Effect of cycloheximide on the reaction of puromycin with polysome-bound peptidyl-tRNA. FEBS lett. *8*:249.

Baliga, B.S., Pronczuk, A.W., and Munro, H.N. 1969. Mechanism of cycloheximide inhibition of protein synthesis in a cell-free system prepared from rat liver. J. Biol. Chem. *244*:4480.

Birbeck, M.S.C., and Mercer, E.H. 1961. Cytology of cells which synthesize protein. Nature *189*:558.

Blobel, G., and Potter, V.R. 1967. Studies on free and membrane-bound ribosomes in rat liver. I. Distribution as related to total cellular tRNA. J. Mol. Biol. *26*:279.

Blobel, G., and Sabatini, D. 1971. Dissociation of mammalian polyribosomes into subunits by puromycin. Proc. Nat. Acad. Sci. USA *68*:390.

Bollen, A., Petre, J., and Grosjean, H. 1972. Direct biochemical approach of the structural heterogeneity of 30S ribosomes from *Escherichia coli*. FEBS lett. *24*:327.

Borun, T.W., Scharff, M., and Robbins, E. 1967. Rapidly labeled polyribosome-associated RNA having the properties of histone messenger. Proc. Nat. Acad. Sci. *58*:1977.

Brenner, S., Jacob, F., and Meselson, M. 1961. An unstable intermediate carrying information from genes to ribosomes for protein synthesis. Nature *190*:576.

Bulova, S.I., and Burka, E.R. 1970. Biosynthesis of nonglobin protein by membrane-bound ribosomes in reticulocytes. J. Biol. Chem. *245*:4907.

Busiello, E., Di Girolamo, M., and Felicetti, F. 1971. Role of mammalian ribosomal subunits and elongation factors in poly(U)-direct protein synthesis. Biochem. Biophys. Acta *228*:289.

Chao, F.C., and Schachman, N.K. 1956. The isolation and characterization of a macromolecular ribonucleoprotein from yeast. Arch. Biochem. Biophys.*61*:220.

Cold Spring Harbour N Y Laboratory of Quantitative Biology. 1969. Cold Spring Harbor Symp. Quant. Biol. *34*.

Collins, J.F., Moon, H.M., and Maxwell, E.S. 1972. Multiple forms and some properties of aminoacyltransferase I (Elongation factor I) from rat liver. Biochemistry,*11*:4187.

Craig, N., Kelley, D.E., and Perry, R.P. 1971. Lifetime of the messenger RNAs which code for ribosomal proteins in L-cells. Biochem. Biophys. Acta *246*:493.

Crick, F.H.C. 1958. On protein synthesis *The biological replication of macromolecules*, Symposium of the Society for experimental biology. XII, p 138, Cambridge University Press, London.

Crick, F.H.C., Barnett, L., Brenner, S., Watts-Tobin, R.J. 1961. General nature of the genetic code for proteins. Nature *192*:1227.

Darnell, J.E., Philipson, L., Wall, R., and Adesnik, M. 1971. Polyadenylic acid sequences:role in conversion of nuclear RNA into messenger RNA. Science *174*:507.

Delauney, J., Mathier, C., and Schapira, G. 1972. Eukaryotic ribosomal proteins. Interspecific and intraspecific comparisons by two-dimensional polyacrylamide-gel electrophoresis. Eur. J. Biochem. *31*:561.

Edmonds, M., Vaughan, M.H., and Nakazoto, H. 1971. Polyadenylic acid sequences in the heterogeneous nuclear RNA and rapidly-labeled polyribosomal RNA of Hela cells:possible evidence for a precursor relationship. Proc. Nat. Acad. Sci. *68*:1336.

Eisenstadt, J.M., and Brawerman, G. 1967. The role of the native subribosomal particles of *Escherichia coli* in polypeptide chain initiation. Proc. Nat. Acad. Sci. USA *58*:1560.

Faber, A.J., and Tamaoki, T. 1972. Isolation of active ribosomal subunits from L5178Y mouse lymphoma cells. Arch. Biochem. Biophys. *149*:289.

Falvey, A.K., and Staehelin, T., 1970. Structure and function of mammalian ribosomes I. Isolation and characterization of active liver ribosomal subunits. J. Mol. Biol. *53*:1.

Fogel, S., and Sypherd, P.S. 1968. Chemical basis for heterogeneity of ribosomal proteins. Proc. Nat. Acad. Sci. USA *59*:1329.

Gamow, G. 1954. Possible relation between deoxyribonucleic acid and protein structure. Nature *173*:318.

Ganoza, M.C., and Williams, C.A. 1969. *In vitro* synthesis of different categories of specific protein by membrane-bound and free ribosomes. Proc. Nat. Acad. Sci. *63*:1370.

Gierer, A. 1958. Grosse und struktur der ribosenucleinsaure das Tabakmosiakvirus. Z. Naturforsch *136*:477.

Gilbert, B.E., and Johnson, T.C. 1972. The use of aminoacyl-tRNA to measure polypeptide synthesis by ribosomes isolated from neonatal and adult mouse brain tissue. Biochem. Biophys. Res. Commun. *46*:2034.

Gilbert, J.M., and Anderson, W.F. 1970. Cell-free hemoglobin synthesis.II. Characteristics of the transfer ribonucleic acid-dependent assay system. J. Biol. Chem. *245*:2342.

Gilbert, W., 1963. Polypeptide synthesis in *Escherichia coli*. I. Ribosomes and the active complex. J. Mol. Biol. *6*: 374.

Girolamo, M.D. and Cammarano, R. 1968. The protein composition of ribosomes and ribosomal subunits from animal tissues. Electrophoretic studies. Biochim. Biophys. Acta. *168*:181.

Godchaux, W., III, Adamson, S.D., and Herbert, E. 1967. Effects of cycloheximide on polyribosome function in reticulocytes. J. Mol. Biol. *27*:57.

Grollman, A.P., and Stewart, M.L. 1968. Inhibition of the attachment of messenger ribonucleic acid to ribosomes. Proc. Nat. Acad. Sci. USA *61*:719.

Grummt, F. 1970. A protein fraction from rat liver ribosomes stimulating initiation of polyphenylalanine synthesis at low Mg^{2+} concentration. Acta Biol. Med. Germ. *24*:K55.

Grummt, F., and Bielka, H. 1971. Isolation of a protein fraction from rat liver ribosomes initiating polyphenylalanine synthesis at low Mg^{2+} ions-concentrations. Eur. J. Biochem. *21*:210.

Gurdon, J.B., Lane, C.D., Woodland, H.R., and Marboix, G. 1971. Eggs and oocytes for the study of messenger RNA in its translation in living cells. Nature *233*:177.

Guthrie, C., and Nomura, M. 1968. Initiation of protein synthesis: a critical test of the 30S subunit model. Nature *219*:232.

Hamilton, M. 1967. The molecular weight of the 30-S RNA of Jensen Sarcoma ribosomes as determined by equilibrium centrifugation. Biochim. Biophys. Acta *134*:473.

Hamilton, M.G., and Petermann, M.L. 1959. Ultracentrifugal studies on ribonucleoprotein from rat liver microsomes. J. Biol. Chem. *234*:1441.

Hamilton, M.G., and Ruth, M.E. 1969. The dissociation of rat liver ribosomes by ethylenediaminetetraacetic acid; molecular weight, chemical composition buoyant densities. Biochemistry *8*:851.

Hardy, S.J.S., Kurland, C.G., Voynow, P., and Mora, G. 1969. The ribosomal proteins of *Escherichia coli* I. Purification of the 30S ribosomal proteins. Biochemistry *8*:2897.

Haselkorn, R., and Rothman-Denes, L.B. 1973. Protein synthesis. Ann.Rev. Biochem. *42*:397.

Henshaw, E.C., Guiney, D.G., and Hirsch, C.A. 1973. The ribosome cycle in mammalian protein synthesis. I. The place of monomeric ribosomes and ribosomal subunits in the cycle. J. Biol. Chem. *248*:4367.

Heywood, S.M., 1970. Specificity of mRNA binding factor in Eukaryotes. Proc. Nat. Acad. Sci. USA *67*:1782.

Heywood, S.M., and Nwagwu, M. 1969. Partial characterization of presumptive myosin messenger ribonucleic acid. Biochemistry *8*:3839.

Hill, H.Z., Wilson, S.H., and Hoagland, M.B. 1972. Patterns of albumin and general protein synthesis in rat liver as revealed by gel electrophoresis. Biochem. Biophys. Acta *269*:477.

Hille, M.B., Miller, M.J., Iwasaki, K., and Wahba, A.J. 1967. Translation of the genetic message, VI. The role of ribosomal subunits in binding of formylmethionyl–tRNA and its reaction with puromycin. Proc. Nat. Acad. Sci. USA *58*:1652.

Hoagland, M.B. and Keller, E.B., and Zamecnik, P.C. 1956. Enzymatic carboxyl activation of amino acids. J. Biol. Chem. *218*:345.

Hoagland, M.B., Zamecnik, P.C., and Stephenson, M.L. 1957. Intermediate reactions in protein biosynthesis Biochem. Biophys. Acta *24*:215.

Housman, D., Pemberton, R., and Taber, R. 1971. Synthesis of α and β chains of rabbit hemoglobin in a cell-free extract from Krebs II ascites cells. Proc. Nat. Acad. Sci. USA *68*:2716.

Huynh-Van-Tan, Delouney, J., and Scharira, G. 1971. Eukaryotic ribosomal proteins. Two-dimensional electrophoretic studies. FEBS lett. *17*:163.

Jacob, F., and Monod, J. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. *3*:318.

Jacobs-Lorena, M., Baglioni, C., and Borun, T.W. 1972. Translation of messenger RNA for histones from Hela cells by a cell-free extract from mouse ascites tumor. Proc. Nat. Acad. Sci. USA *69*:2095.

James, E., and Morrison, J.F., 1966. The reaction of nucleotide substrate analogues with adenosine triphosphate- creatine phosphotransferase. J. Biol. Chem., *241*:4758.

Kaempfer, R. 1968. Ribosomal subunit exchange during protein synthesis. Proc. Nat. Acad. Sci. USA *61*:106.

Kaempfer, R. 1970. Dissociation of ribosomes on polypeptide chain termination and origin of single ribosomes. Nature *228*:534.

Kaempfer, R. 1970. Initiation factor IF-3: a specific inhibitor of ribosomal subunit association. J. Mol. Biol. *71*:583.

Kaempfer, R. 1971. Control of single ribosome formation by an initiation factor for protein synthesis. Proc. Nat. Acad. Sci. USA *68*:2458.

Kates, J. 1970. Transcription of the Vaccinia Virus Genome and the occurrence of polyriboadenylic acid sequences in messenger RNA. Cold Spring Harbour Symp. Quant. Biol. *35*:743.

Kitos, P.A., Saxon, G., and Amos, H. 1972. The isolation of polyadenylate with unreacted cellulose. Biochem. Biophys. Res. Commun. *47*:1426.

Kuff, E.L., and Zeigel, R.F. 1960. Cytoplasmic ribonucleoprotein components of the Novikoff Hepatoma. J. Biophys. Biochem. Cytol. *7*:465.

Lamfrom, H., and Glowacki, E.R. 1962. Controlled dissociation of rabbit reticulocyte ribosomes and its effect on hemoglobin synthesis. J. Mol. Biol. *5*:97.

Lawford, G.R. 1969. The effect of incubation with puromycin on the dissociation of rat liver ribosomes into active subunits. Biochem. Biophys. Res. Commun. *37*:143.

Leader, D.P., Klein-Bremhaar, H., Wool, I.G., and Fox, A. 1972. Distribution of initiation factors in cell fractions from mammalian tissues. Biochem. Biophys. Res. Commun. *46*:215.

LeBleu, B., Marbaix, G., Werenne, J., Burny, A., and Huez, G. Effect of aurintricarboxylic acid and of NaF on the binding of globin messenger RNA to reticulocyte 40S ribosomal subunits. Biochem. Biophys. Res. Commun. *40*:731.

Lee, S.Y., Mendecki, J., and Brawerman, G. 1971. A polynucleotide segment rich in adenylic acid in the rapidly-labeled polyribosomal RNA component of mouse sarcoma 180 ascites cells. Proc. Nat. Acad. Sci. USA *68*:1331.

Lengyel, P., and Soll, D. 1969. Mechanism of protein biosynthesis. Bacteriological Reviews *33*:264.

Lerman, M.I., Spirin, A.J., Gavrilova, L.P. and Golov, V.F. 1966. Studies on the structure of ribosomes.II.Stepwise dissociation of protein from ribosomes by caesium chloride and the re-assembly of ribosome-like particles. J. Mol. Biol. *15*:268.

Lockard, R.E. and Lingrel, J.B. 1969. The synthesis of mouse hemoglobin β-chains in a rabbit reticulocyte cell-free system programmed with mouse reticulocyte 9S RNA. Biochem. Biophys. Res. Commun. *37*:204.

Low, R.B., and Rich, A. 1973. Myoglobin biosynthesis in the embryonic chick. Biochemistry *12*:4555.

Low, R.B., and Wool, I.G. 1967. Mammalian ribosomal protein: analysis by electrophoresis on polyacrylamide gel. Science *155*:330.

Lowry, O.H., Rosebrough, N.J. Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*:265.

Lucas-Lenard, J., and Lipmann, F. 1966. Separation of three microbial amino acid polymerization factors. Proc. Nat. Acad. Sci. USA *55*:1562.

Lucas-Lenard, J., and Lipmann, F. 1971. Protein biosynthesis. Ann. Rev. Biochem. *40*:409.

MacInnes, J.W., 1972. Differences between ribosomal subunits from brain and those from other tissues. J. Mol. Biol. *65*:157.

Mangiarotti, G., and Schlessinger, D. 1967. Polyribosome metabolism in *Escherichia coli* II. Formation and lifetime of messenger RNA molecules, ribosomal subunit couples and polyribosomes. J. Mol. Biol. *29*, 395.

Marcker, K., and Sanger, F. 1964. N-formyl-methionyl-S-RNA. J. Mol. Biol. *8*:835.

Marcot-Queiroz, J., and Monier, R. 1965. Interactions entre RNAs ribosomes. Bull. Soc. Chim. Biol. *47*:1627.

Marcot-Queiroz, J., and Monier, R. 1965. Interactions between RNAs from *Escherichia coli* ribosomes. J. Mol. Biol. *14*,:490.

Martin, T.E., Rolleston, F.S., Low, R.B., and Wool, I.G. 1969. Dissociation and reassociation of skeletal muscle ribosomes. J. Mol. Biol. *43*:135.

Martin, T.E., and Wool, I.G. 1968. Formation of active hybrids from subunits of muscle ribosomes from normal and diabetic rats. Proc. Nat. Acad. Sci. *60*:569.

Martin, T.E., and Wool, I.G. 1969. Active hybrid 80S particles formed from subunits of rat, rabbit and protozoan (*Tetrahymena pyriformis*) ribosomes. J. Mol. Biol. *43*:151.

Mathews, M.B. 1972. Further studies on the translation of globin mRNA and encephalomyocarditis virus RNA in a cell-free system from Krebs II ascites cells. Biochim. Biophys. Acta *272*:108.

Mathews, M.B., Osbern, M., Berns, A.J.M., and Bloemendal, H. 1972. Translation of two messenger RNAs from lens in a cell-free system from Krebs II ascites cells. Nature (London) New Biol. *236*:5.

Mathews, M.B., Osborn, M., and Lingrel, J.B. 1971. Translation of globin messenger RNA in a heterologous cell-free system. Nature New Biol. *233*:206.

McConkey, E.H. 1967. The fractionation of RNAs by sucrose gradient centrifugation. Methods in Enzymology, Vol. XII A, Academic Press, New York and London.

McKeehan, W., and Hardesty, B. 1969. The mechanism of cycloheximide inhibition of protein synthesis in rabbit reticulocytes. Biochem. Biophys. Res. Commun. *36*:625.

McKeehan, W.L., and Hardesty, B. 1969. Purification and partial characterization of the aminoacyl transfer ribonucleic acid binding enzyme from rabbit reticulocytes. J. Biol. Chem. *244*:4330.

McKeehan, W., Irvin, J., and Hardesty, B. 1970. A factor in the salt wash of reticulocyte ribosomes that can interact with acetylphenylalanyl-tRNA. Biochem. Biophys. Res. Commun. *41*:757.

Means, A.R., Comstock, J.P., and O'Malley, B.W. 1971. Isolation of protein factors from oviduct polysomes which stimulate protein synthesis. Biochem. Biophys. Res. Commun. *45*:759.

Mechler, B., and Mach, B. 1971. Preparation and properties of ribosomal subunits from mouse plasmocytoma tumors. Eur. J. Biochem. *21*:552.

Mendecki, J., Lee, S.Y., and Brawerman, G. 1972. Characteristics of the polyadenylic acid segment associated with messenger ribonucleic acid in mouse sarcoma 180 ascites cells. Biochemistry *11*:792.

Metafora, S., Terada, M., Dow, L.W., Marks, P.A., and Bank, A. 1972. Increased efficiency of exogenous messenger RNA translation in a Krebs ascites cell lysate. Proc. Nat. Acad. Sci. USA *69*:1299.

Miller, R.L., and Schweet, R. 1968. Isolation of a protein fraction from reticulocyte ribosomes required for *de novo* synthesis of hemoglobin. Arch. Biochem. Biophys. *125*:632.

Moldave, K. 1968. Aminoacyl transfer to ribosomal protein in the rat liver system; Methods in Enzymology, Vol XII B, Academic Press, New York and London.

Moore, P.B. 1966. Studies on the mechanism of messenger ribonucleic acid attachment to ribosomes. J. Mol. Biol. *22*:145.

Morgan, R.S., Greenspan, C., and Cunningham, B. 1963. Dissociation of yeast ribosomes by papain. Biochim. Biophys. Acta *68*:642.

Morgan, A.R., Wells, R.D., Khorana, H.G. 1966. Studies on polynucleotides LIX. Further codon assignments from amino acid incorporations directed by ribopolynucleotides containing repeating trinucleotide sequences. Proc. Nat. Acad. Sci. USA *56*:1899.

Munro, H.N., Baliga, B.S., and Pronczuk, A.W. 1968. *In vitro* inhibition of peptide synthesis and GTP hydrolysis by cycloheximide and reversal of inhibition by glutathione. Nature, London *219*:944,

Mutolo, V. Giudice, G., Hopps, V., and Donatuti, G. 1967. Species specificity of embryonic ribosomal proteins. Biochim. Biophys. Acta *138*:214.

Nirenberg, M., and Leder, P. 1964. RNA codewords and protein synthesis. The effect of trinucleotides upon the binding of sRNA to ribosomes. Science *145*:1399.

Nirenberg, M.W., and Matthaei, J.H. 1961. The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides. Proc. Nat. Acad. Sci. USA *47*:1588.

Noll, H. 1967. "Techniques in protein biosynthesis" Volume 2, Page 112, New York Academic Press.

Nomura, M. and Lowry, C.V. 1967. Phage F_2 RNA-directed binding of formylmethionyl-tRNA to ribosomes and the role of 30S ribosomal subunits in initiation of protein synthesis. Proc. Nat. Acad. Sci. USA *58*:946.

Nomura, M., Lowry, C.V., and Guthrie, C. 1967. The initiation of protein synthesis: joining of the 50S ribosomal subunit to the initiation complex. Proc. Nat. Acad. Sci. USA *58*:1487.

Nonomura, Y., Blobel, G., and Sabatini, D. 1971. Structure of liver ribosomes studied by negative staining. J. Mol. Biol. *60*:303.

Obrig, T.G., Culp, W., McKeehan, W., and Hardesty, B. 1971. The mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J. Biol. Chem. *246*:174.

Ohtaka, Y., and Uchida, K. 1963. The chemical structure and stability of yeast ribosomes. Biochim. Biophys. Acta *76*:94.

Otaka, E., Itoh, T., Osawa, S. 1968. Ribosomal proteins of bacterial cells: strain- and species-specificity. J. Mol. Biol. *33*:93.

Palacios, R., Schimke, R.T. 1973. Indentification and isolation of ovalbumin-synthesizing polysomes. J. Biol. Chem. *248*:1424.

Palacios, R., Sullivan, D., Summers, N.M., Kiely, M.L., and Schimke, R.T. 1973. Purification of ovalbumin messenger ribonucleic acid by specific immunoadsorption of ovalbumin-synthesizing polysomes and millipore partition of ribonucleic acid. J. Biol. Chem. *248*:540.

Palade, G., and Siekevitz, P. 1956. Liver microsomes. An integrated morphological and biochemical study. J. Biophys. Biochem. Cytol. *2*:171.

Perry, R.P., LaTorre, J., Kelly, D.E., and Greenberg, J.R. 1972. On the lability of poly(A) sequences during extraction of messenger RNA from polyribosomes. Biochem. Biophys. Acta *262*:220.

Pestka, S., and Nirenberg, M. 1966. Regulatory mechanisms and protein synthesis. X.Codon recognition on 30S ribosomes. J. Mol. Biol. *21*:145.

Petermann, M.L. 1963. "The physical and chemical properties of ribosomes". Elsevier, Amsterdam.

Petermann, M.L., and Pavlovec, A. 1963. Studies on ribonucleic acid from rat liver ribosomes. J. Biol. Chem. *238*:3717.

Petermann, M.L., and Pavlovec, A. 1966. The subunits and structural ribonucleic acids of Jensen Sarcoma ribosomes. Biochim. Biophys. Acta *114*:264.

Petermann, M.L., and Pavlovec, A. 1969. Effects of magnesium and formaldehyde on the sedimentation behavior of rat liver ribosomes. Biopolymers *7*:73.

Petermann, M.L., and Pavlovec, A. 1971. Dissociation of rat liver ribosomes to active subunits by urea. Biochemistry *10*:2770.

Peters, T. Jr. 1962. The biosynthesis of rat serum albumin II. Intracellular phenomena in the secretion of newly formed albumin. J. Biol. Chem. *237*:1186.

Peters, T. Jr. 1970. Serum albumin. Advances in Clinical Biochemistry. *13*:37.

Picciano, D.J., Prichard, P.M., Merrick, W.C., Shafritz, D.A., Graff, H., Crystal, R.G., and Anderson, W.F. Isolation of protein synthesis initiation factors from rabbit liver. J. Biol. Chem. *248*:204.

Prichard, P.M., Picciano, D.J., Laycock, D.G., and Anderson, W.F. 1971. Translation of exogenous messenger RNA for hemoglobin on reticulocyte and liver ribosomes. Proc. Nat. Acad. Sci. USA *68*:2752.

Raeburn, S., Collins, J.F., Moon, H.M., and Maxwell, E.S. 1971. Aminoacyltransferase II from rat liver. I. Purification and enzymatic properties. J. Biol. Chem. *246*:1041.

Randall-Hazelbauer, L.L., and Kurland, G.G. 1972. Identification of three 30S proteins contributing to the ribosomal A site. Mol. Gen. Genetics *115*:234.

Rao, P., and Moldave, K. 1969. Interaction of polypeptide chain elongation factors with rat liver ribosomal subunits. J. Mol. Biol. *46*:447.

Rao, S.S., and Grollman, A.P. 1967. Cycloheximide resistance in yeast: a property of the 60S ribosomal subunit. Biochem. Biophys. Res. Commun. *29*:696.

Redman, C.M. 1969. Biosynthesis of serum proteins and ferritin by free and attached ribosomes of rat liver. J. Biol. Chem. *244*:4308.

Rhoads, R.E., McKnight, G.S., and Schimke, R.T. 1971. Synthesis of ovalbumin in rabbit reticulocyte cell-free system programmed with hen oviduct ribonucleic acid. J. Biol. Chem. *246*:1.

Rosenfeld, G.C., Comstock, J.P., Means, A.R., and O'Malley, B.W. 1972. A rapid method for the isolation and partial purification of specific eucaryotic messenger RNAs. Biochem. Biophys. Res. Commun. *47*:387.

Sabol, S., Sillero, M.A.G., Iwasaki, K., and Ochoa, S. 1970. Purification and properties of initiation factor F_3. Nature *228*:1269.

Schechter, I. 1973. Biologically and chemically pure mRNA coding for a mouse immunoglobin L-chain prepared with the aid of antibodies and immobilized oligothymidine. Proc. Nat. Acad. Sci. USA *70*:2256.

Schneir, M., and Moldave, K. 1968. The isolation and biological activity of multiple forms of aminoacyl transferase I of rat liver. Biochem. Biophys. Acta *166*:58.

Schochetman, G. Perry, R.P., 1972. Early appearance of histone messenger RNA in polyribosomes of cultured L cells. J. Mol. Biol. *63*:591.

Schutz, G., Beato, M., and Feigelson, P. 1972. Isolation of eukaryotic messenger RNA on cellulose and its translation *in vitro*. Biochem. Biophys. Res. Commun. *49*:680.

Schutz, G., Beato, M., Feigelson, P. 1973. Messenger RNA for hepatic tryptophan oxygenase: its partial purification, its translation in a heterologous cell-free system, and its control by glucocorticoid hormones. Proc. Nat. Acad. Sci. USA *70*:1218.

Shafritz, D.A. 1974. Protein synthesis with messenger ribonucleic acid fractions from membrane-bound and free liver polysomes. J. Biol. Chem. *249*:81.

Shafritz, D.A., and Anderson, W.F. 1970. Factor dependent binding of methionyl-tRNAs to reticulocyte ribosomes. Nature *227*:918.

Shafritz, D.A., and Anderson, W.F. 1970. Isolation and partial characterization of reticulocyte factors M_1 and M_2. J. Biol. Chem. *245*:5553.

Shafritz, D.A., Prichard, P.M., Gilbert, J.M., and Anderson, W.F. 1970. Separation of two factors, M_1 and M_2, required for poly(U) dependent polypeptide synthesis by rabbit reticulocyte ribosomes at low magnesium ion concentration. Biochem. Biophys. Res. Commun. *38*:721.

Shakulov, R.S., Ajtkhozhin, M.A., and Spirin, A.S. 1962. The latent degradation of ribosomes. Biochemistry *27*:631.

Sheldon, R., Jurale, C., and Kates, J. 1972. Detection of polyadenylic acid sequences in viral and eukaryotic RNA. Proc. Nat. Acad. Sci. *69*:417.

Siekevitz, P. 1952. Uptake of radioactive alanine *in vitro* into the proteins of rat liver fractions. J. Biol. Chem. *195*:549.

Siekevitz, P., and Palade, G. 1958. A cytochemical study on the pancreas of the guinea pig. I. Isolation and enzymatic activities of cell fractions. II. Functional variation in the enzymatic activity of microsomes. III. *In vivo* incorporation of leucine-1-C^{14} into the proteins of cell fractions. J. Biophys. Biochem. Cytol. *4*:203,309,557.

Siekevitz, P., and Palade, G.E. 1960. A cytochemical study on the pancreas of the guinea pig. V. *In vivo* incorporation of Leucine-1-C^{14} into the chymotrypsinogen of various cell fractions. J. Biophys. Biochem. Cytol. *7*:619.

Siekevitz, P., and Palade, G.E. 1962. Cytochemical study on the pancreas of the guinea pig. VII Effects of spermine on ribosomes. J. Cell. Biol. *13*:217.

Skogerson, L., and Moldave, K. 1968b. Characterization of the interaction of aminoacyltransferase II with ribosomes. J. Biol. Chem. *243*:5354.

Smith, A.E., and Marcker, K.A. 1970. Cytoplasmic methionine transfer RNAs from eukaryotes. Nature *226*:607.

Spitnik-Elson, P. 1965. The preparation of ribosomal protein from *Escherichia coli* with lithium chloride and urea. Biochem. Biophys. Res. Commun. *18*:557.

Subramanian, A.R., and Davis, B.D. 1970. Activity of initiation factor F_3 in dissociating *Escherichia coli* ribosomes. Nature *228*:1273.

Takagi, M., Ogata, K. 1968. Direct evidence for albumin biosynthesis by membrane bound polysomes in rat liver. Biochem. Biophys. Res. Commun. *33*:55.

Takagi, M., Tanaka, T., and Ogata, K. 1970. Functional differences in protein synthesis between free and bound polysomes of rat liver. Biochim. Biophys. Acta *217*:148.

Tamaoki, T., and Miyazawa, F. 1967. Dissociation of *Escherichia coli* ribosomes by sulfhydryl reagents. J. Mol. Biol. *23*:35.

Tashiro, Y., and Morimoto, T. 1966. Sedimentation studies on the interaction of ribosomal subunits from Liver. Biochim. Biophys. Acta *123*:523.

Tashiro Y., and Siekevitz, P. 1965. Ultracentrifugal studies on the dissociation of hepatic ribosomes. J. Mol. Biol. *11*:149.

Tashiro, Y., and Yphantis, D.A. 1965. Molecular weights of hepatic ribosomes and their subunits. J. Mol. Biol. *11*:174.

Taylor, J.M., and Schimke, R.T. 1973. Synthesis of rat liver albumin in a rabbit reticulocyte cell-free protein-synthesizing system. J. Biol.Chem. *248*:7661.

Temple, G.F., Housman, D.E. 1972. Separation and translation of the mRNAs coding for α and β chains of rabbit globin. Proc. Nat. Acad. Sci. USA *69*:1574.

Terao, K., and Ogata, K. 1970. Preparation and some properties of active subunits from rat liver ribosomes. Biochem. Biophys. Res. Commun. *38*:80.

Thompson, W.C., Buzash, E.A. and Heywood, S.M. 1973. Translation of myoglobin messenger ribonucleic acid. Biochemistry *12*:4559.

Tissieres, A., and Watson, J.D. 1958. Ribonucleoprotein particles from *Escherichia coli*. Nature *182*:778.

Traub, P., and Nomura, M. 1969. Studies on the assembly of ribosomes *in vitro*. Cold Spring Harbour Symp. Quant. Biol. *34*:63.

Uenoyama, K., and Ono, T. 1972. Nascent catalase and its messenger RNA on rat liver polyribosomes. J. Mol. Biol. *65*:75.

Van Duin J., and Kurland C. 1970. Functional heterogeneity of the 30S ribosomal subunit of *E. coli*. Mol. Gen. Genetics *109*:169.

Von Der Decken, A., Ashby, P., McIlreavy, D., and Campbell, P.M. 1970. The reversible dissociation and activity of ribosomal subunits of liver and and skeletal muscle from rats. Biochem.J. *120*:815.

Wang, C.S., Naso, R.B., and Arlinghaus, R.B. 1972. Factor-dependent binding of Rauscher leukemia virus RNA to ribosomes. Biochem.Biophys. Res. Commun. *47*:1290.

Watson, J.D. 1964. The synthesis of proteins upon ribosomes. Bull. Soc. Chem. Biol. *46*:1339.

Webster, R.E., and Zinder, N.D. 1969. Fate of the message-ribosome complex upon translation of termination signals. J. Mol. Biol. *42*:425.

Wigle, D.T., and Smith A.E. 1973. Specificity in initiation of protein synthesis in a fractionated mammalian cell-free system. Nature New Biology *242*:136.

Woodley, C.L., Chen, Y.C., Bose, K.K., and Gupta, N.K. 1972. Protein synthesis in rabbit reticulocytes; characteristics of peptide chain initiation factors. Biochem. Biophys. Res. Commun. *46*:839.

Zamir, A., Holley, R.W., and Marquisee, M. 1965. Evidence for the occurrence of a common pentanucleotide sequence in the structures of transfer ribonucleic acids. J. Biol. Chem. *240*:1267.

Zillig, W., Krone, W., and Albers, M. 1959. Utersuchugen zur biosynthese der proteine, III^1. Hoppe-Seyler's A. physio Chem. *317*:131.